

## A Device

### **Related applications**

This application is a continuation-in-part of US application Serial No. 09/568,254, filed 10 May 2000. This application also claims priority from U.S. provisional application 60/271,497, filed February 26, 2001, and UK applications 0101315.0, filed 18 Jan 2001, and 0107093.7, filed March 21, 2001. Reference is also made to U.S. Patent Application Serial No. 09/742,520, filed December 20, 2000, as a divisional of U.S. application Serial No. 09/521,700, filed March 9, 2000, which in turn is a divisional of application Serial no. 08/594,164, filed January 31, 1996, now U.S. Patent No. 6,090,625. Each of the foregoing applications and patents, and each document cited or referenced in each of the foregoing applications and patents, including during the prosecution of each of the foregoing applications and patents ("application cited documents") and any manufacturer's instructions or catalogues for any products cited or mentioned in each of the foregoing applications and patents and in any of the application cited documents, are hereby incorporated herein by reference. Furthermore, all documents cited in this text, and all documents cited or referenced in documents cited in this text, and any manufacturer's instructions or catalogues for any products cited or mentioned in this text, are hereby incorporated herein by reference.

### **Technical field of the invention**

The present invention relates to a device. In more detail, the present invention relates to a device for preparing an undifferentiated cell. In particular, but not exclusively, the present invention relates to a device for preparing an undifferentiated cell from a more committed cell. In a further aspect, the present invention also relates to a method of forming undifferentiated cells. The device can also remotely communicate with the producer/distributor/manufacturer/call centre/service centre and the like, for example to remotely order new agent and/or to confirm that operations are being or have been performed correctly. Thus, the invention also relates to methods of doing business involving the device and uses thereof, for example remotely communicating with a producer/distributor/manufacturer/call centre/service centre and the like and the producer/distributor/manufacturer/call centre/service centre and the like responding to such communications (for example, receiving and filling orders, receiving and/or processing and/or responding to data/information regarding operations and/or correctness thereof).

### **Background to the invention**

Differentiation is a process whereby structures and functions of cells are progressively committed to give rise to more specialised cells, such as the formation of T cells or B cells from immature haematopoietic precursors. Therefore, as the cells become more committed, they become more specialised. In the majority of mammalian cell types, cell differentiation is a one-way process leading ultimately to terminally differentiated cells. However, although some cell types persist throughout life without dividing and without being replaced, many cell types do continue to divide during the lifetime of the organism and undergo renewal. This may be by simple division (e.g. liver cells) or, as in the case of cells such as haematopoietic cells and epidermal cells, by division of relatively undifferentiated stem cells followed by commitment of one of the daughter cells to a programme of subsequent irreversible differentiation. All of these processes, however,

have one feature in common: cells either maintain their state of differentiation or become more differentiated. They do not become undifferentiated or even less differentiated.

5 Retrodifferentiation is a process whereby structures and functions of cells are progressively changed to give rise to less specialised cells. Some cells naturally undergo limited reverse differentiation (retrodifferentiation) *in vivo* in response to tissue damage. For example, liver cells have been observed to revert to an enzyme expression pattern similar to the foetal enzymic pattern during liver regeneration (Curtin and Snell, 1983, Br. J. Cancer, Vol. 48; 495-505).

10 In WO96/23870 it was shown that it was possible to treat differentiated cells so that they became undifferentiated cells, including stem cells. These undifferentiated cells were capable of proliferating and giving rise to redifferentiated progeny of the same lineage or any other lineage. In the case of retrodifferentiated haematopoietic cells, these stem cells  
15 are pluripotent and can give rise to more than one cell lineage. The seminal finding of WO96/23870 were completely unexpected.

The clinical implications of this finding are enormous. Stem cells are extremely difficult to obtain from human patients. They are typically obtained from umbilical tissue, bone marrow or blood where they are present in only very small amounts. However, the  
20 present invention provides a device for producing stem cells from more committed cells, in particular by the process of retrodifferentiation.

US 6,087,168 discloses a method of transdifferentiating epidermal cells into neuronal cells, in which method epidermal cells are dedifferentiated or retrodifferentiated with  
25 an appropriate medium. Likewise, Lake JA *et al* Journal of Cell Science 113, 556-566 (2000) and Rathjen J *et al* Journal of Cell Science 112, 601-612 (1999) disclose the retrodifferentiation of embryonic stem (ES) cells into early primitive ectoderm-like (EPL) cells, in response to two separable factors.

## Summary of the invention

In a broad aspect there is provided a device for preparing an undifferentiated cell, wherein the device comprises means for allowing a more committed cell to retrodifferentiate into an undifferentiated cell.

None of the prior art documents (such as US 6,087,168, Lake *et al* or Rathjen *et al*) discloses a device suitable for use in the preparation of undifferentiated cells. Although the preparation of undifferentiated cells can be carried out by a person skilled in the art without the use a device according to the present invention, the final product tends to be inconsistent as each time the procedure is conducted the results depend upon the skills and experience of the person conducting the procedure. In addition, the "hands-on" time required to manually prepare undifferentiated cells is large and thus is not cost-effective for a laboratory.

In one specific embodiment there is provided a device for forming and/or increasing the relative number of undifferentiated cells in a cell population including committed cells, which device comprises a chamber, means for introducing into said chamber a cell population including committed cells, means for introducing into said chamber retrodifferentiation means that are capable of causing a committed cell to retrodifferentiate into an undifferentiated cell, and incubation means for incubating said committed cells in the presence of said retrodifferentiation means such that a committed cell retrodifferentiates into an undifferentiated cell.

In another specific embodiment there is provided a device for forming and/or increasing the relative number of undifferentiated cells in a cell population including committed cells, which device comprises a chamber, means for introducing into said chamber a cell population including committed cells, means for introducing into said chamber an agent that causes a committed cell to retrodifferentiate into an undifferentiated cell, and incubation means for incubating said agent and said committed cells such that a committed cell retrodifferentiates into an undifferentiated cell.

Preferably, the agent engages a receptor that mediates capture, recognition or presentation of an antigen at the surface of the committed cells. More preferably, the receptor is an MHC class I antigen or an MHC class II antigen, such as a class I antigen selected from Human-Leukocyte-Associated (HLA)-A receptor, an HLA-B receptor, an HLA-C receptor, an HLA-E receptor, an HLA-F receptor or an HLA-G receptor or a class II antigen selected from an HLA-DM receptor, an HLA-DP receptor, an HLA-DQ receptor or an HLA-DR receptor.

10 Suitably, the receptor may comprise a  $\beta$ -chain having homologous regions. Preferably, the receptor may comprise at least the homologous region of the  $\beta$ -chain of HLA-DR.

Typically, the committed cells are differentiated cells. Preferably, the committed cells are committed haematopoietic cells, preferably cells selected from T-cell colony-forming cells (CFC-T cells), B-cell colony-forming cells (CFC-B cells), eosinophil colony-forming cells (CFC-Eosin cells), basophil colony-forming cells (CFC-Bas cells), granulocyte/monocyte colony-forming cells (CFC-GM cells), megakaryocyte colony-forming cells (CFC-MEG cells), erythrocyte burst-forming cells (BFC-E cells), erythrocyte colony-forming cells (CFC-E cells), T cells and B cells.

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In one preferred embodiment of the present invention, the more committed cell is not a cancer cell. In another preferred embodiment of the present invention, the agent is neither carcinogenic nor capable of promoting cancer growth.

25 In a preferred embodiment, the agent is an antibody to the receptor, such as a monoclonal antibody to the receptor. Specific examples include CR3/43 and monoclonal antibody TAL.1B5.

In one preferred embodiment the agent modulates MHC gene expression. Suitably, the agent may modulate MHC class I<sup>+</sup> and/or MHC class II<sup>+</sup> expression.

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Preferably the agent is used in conjunction with a biological response modifier, such as an alkylating agent, for example alkylating agent that is or comprises cyclophosphamide.

- 5 Preferred undifferentiated cells comprise a stem cell antigen. In a preferred embodiment, the undifferentiated cells are selected from an embryonic stem cell, a pluripotent stem cell, a lymphoid stem cell, a haematopoietic stem cell, a neuronal stem cell, an epithelial stem cell, a mesenchymal stem cell, an endothelial stem cell and a myeloid stem cell. Preferably, the undifferentiated cells are characterised by one or more of following cell
- 10 surface marker designations: CD34<sup>+</sup>, HLA-DR<sup>-</sup>, CD38<sup>-</sup>, CD117, AC133, CD90 and/or CD45<sup>low</sup>. More preferably the undifferentiated cell is CD34<sup>+</sup> and CD38<sup>-</sup>, even more preferably, CD34<sup>+</sup>, CD38<sup>-</sup>, HLA-DR<sup>-</sup> and CD45<sup>low</sup>.

Thus in a preferred embodiment the present invention also provides a device for

15 increasing the relative number of cells having a cell surface marker designation CD34<sup>+</sup> and/or CD38<sup>-</sup> and/or HLA-DR<sup>-</sup> and/or CD45 low and/or CD90 and/or CD117 and/or AC133 in a cell population including committed cells, which device comprises:

- (i) a chamber,
- (ii) means for introducing into said chamber a cell population including committed
- 20 cells,
- (iii) means for introducing into said chamber an agent that operably engages said committed cells; and
- (iv) incubation means operable to incubate said committed cells that are engaged by
- 25 said agent in said chamber such that the relative number of CD34<sup>+</sup> and/or CD38<sup>-</sup> and/or HLA-DR<sup>-</sup> and/or CD45 low and/or CD90 and/or CD117 and/or AC133 cells increases as a result of said engaging.

A device according to the present invention may, optionally, further comprise purification or isolation means for enriching said undifferentiated cells, removing the

30 agent, and/or recovering said undifferentiated cells from the altered cell population. Preferably, the purification means comprises means for identifying a cell surface marker present on the cell surface of the undifferentiated cell or a cell surface marker

present on the surface of the committed cells but substantially absent from the cell surface of the undifferentiated cells. Suitably, the purification means may utilise antibodies raised against the cell surface marker for example. Examples of suitable markers include CD34, CD45 and HLA-DR. By way of example only, a suitable purification means is the CliniMACs/Isolex CD34+ purification system. In a further embodiment of the present invention such a purification or isolation means may be, optionally, provided as a separate means.

A device according to the present invention may, optionally, further comprise upstream negative selection and/or cell enrichment means. Alternatively, the cell population may be, optionally, negatively selected and/or cell enriched prior to insertion into said device.

In another preferred embodiment, the undifferentiated cell of the invention is CD34<sup>+</sup> CD45<sup>-</sup> and negative for markers of haematopoietic lineages.

The more differentiated cells may be of the same lineage as the original committed cells or of different lineage.

Thus, as well as producing undifferentiated cells, a device according to the present invention can be used to convert cells of one lineage to those of another lineage.

Accordingly, in a further aspect the present invention provides a device for inducing in a cell population comprising committed haematopoietic cells of one haematopoietic lineage to become cells of another haematopoietic lineage which device comprises:

- (i) a chamber,
- (ii) means for introducing into said chamber a cell population including committed cells,
- (iii) means for introducing into said chamber an agent that operably engages a receptor that mediates capture, recognition or presentation of an antigen at the surface of said committed haematopoietic cells; and

(iv) incubation means operable to incubate said committed haematopoietic cells that are engaged by said agent in said chamber such that they become cells of another haematopoietic lineage as a result of said engaging.

- 5 Preferably, said committed haematopoietic cells are of a B cell lineage and become cells of another haematopoietic lineage selected from a T cell lineage and a myeloid lineage.

Undifferentiated cells produced by the device of the present invention may be used to manufacture a medicament for the treatment of an immunological disorder or disease.

- 10 Similarly, recommitted cells produced according to the methods of the present invention may be used to manufacture a medicament for the treatment of an immunological disorder or disease.

- The present invention is highly advantageous as it is now possible to easily prepare undifferentiated cells from more committed cells and then use those undifferentiated cells as, or to prepare, medicaments either *in vitro* or *in vivo* or combinations thereof for the treatment of disorders.

- The present invention is also advantageous as it is possible to use the device to commit the undifferentiated cell prepared by retrodifferentiation to a recommitted cell, such as a new differentiated cell, with a view to correcting or removing the original more committed cell or for correcting or removing a product thereof. For example, undifferentiated cells could be used to produce recommitted cells such as the cells lining the alveoli of the lungs, thus creating a mechanism by which damaged or diseased lung tissue can be replaced or repaired (see Le Page, New Scientist 19 December 2000, p 20).

- The term "recommitted cell" means a cell derived from an undifferentiated cell - i.e. a new more committed cell. "More committed" means more differentiated and can easily be determined by reference to known pathways and stages of cell differentiation.

- 30 Most undifferentiated cells and differentiated cells comprise Major Histocompatibility Complex (MHC) Class I antigens and/or Class II antigens. If these antigens are

associated with those cells then they are called Class I<sup>+</sup> and/or Class II<sup>+</sup> cells. Preferably, the more committed cell is capable of retrodifferentiating into a MHC Class I<sup>+</sup> and/or a MHC\ Class II<sup>+</sup> undifferentiated cell.

- 5 Preferably, the more committed cell is capable of retrodifferentiating into an undifferentiated cell comprising a stem cell antigen.

Preferably, the more committed cell is capable of retrodifferentiating into a CD34<sup>+</sup> undifferentiated cell.

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Preferably, the more committed cell is capable of retrodifferentiating into a lymphohaematopoietic progenitor cell.

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Preferably, the more committed cell is capable of retrodifferentiating into a pluripotent stem cell.

Preferably, said increase occurs within 24 hours, preferably 4 to 8 hours (such that any changes cannot be solely accounted for by cell proliferation).

- 20 Typically, the determination of changes in the numbers of undifferentiated cells is performed by monitoring changes in the numbers of cells having cell surface markers characteristic of undifferentiated cells. Examples of suitable cell surface markers include CD34<sup>+</sup>, HLA-DR<sup>-</sup>, CD38<sup>-</sup>, AC133, CD90 and/or CD45 low. Alternatively, or in addition, decreases in the numbers of cells having cell surface markers typical of
- 25 differentiated cells and not undifferentiated cells may be monitored.

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Suitably, a device according to the present invention may, optionally, further comprise tracking means for monitoring the changes in the number of cells having cell surface markers characteristic of undifferentiated cells.

Preferably the committed cells used in the assay are committed haematopoietic cells such as cells selected from CFC-T cells, CFC-B cells, CFC-Eosin cells, CFC-Bas

cells, CFC-GM cells, CFC-MEG cells, BFC-E cells, CFC-E cells, T cells and B cells, more preferably B cells.

5 Preferably a device according to the present invention further comprises one or more, preferably two or more, more preferably three or more, and yet more preferably four or more, of the following features:

- (i) measuring means for measuring the volume of a cell population,
- (ii) counting means (such as a coulter counter, miniaturised if necessary, or other suitable cytometer) for conducting cell counts and thus measuring the cell concentration of a cell population,
- 10 (iii) transfer means for transferring an amount (such as a pre-determined amount) of a cell population from a storage container to the chamber, which transfer means may optionally comprise a pump for instance,
- (iv) calculator means for calculating the volume of agent to be added to the chamber, the volume of agent being dependent on both volume and cell concentration of the cell population,
- 15 (v) further transfer means for transferring a volume (such as a calculated volume) of agent to the chamber, which further transfer means may optionally comprise for instance a syringe driven by a motor, a stepper-motor for example,
- 20 (vi) carbon dioxide control means (as part of the incubation means for instance) for controlling the concentration of carbon dioxide in the chamber,
- (vii) temperature control means (as part of the incubation means for instance) for controlling the temperature in the chamber,
- (viii) mixing means (as part of the incubation means for instance) for mixing the cell population and agent within the chamber,
- 25 (ix) timing means (as part of the incubation means for instance) for timing the incubation period and, optionally, display means for displaying to the user the remaining time period and/or alarm means for alerting the user of completion of the incubation period,
- 30 (x) harvesting means for harvesting cells from the chamber, in particular for harvesting the undifferentiated cells from the chamber,

- (xi) removal means for removing a population of cells, comprising undifferentiated cells, from the chamber into a storage container; the removal means may comprise a pump for example, and
- (xii) sealing means for sealing a storage container comprising a population of cells comprising undifferentiated cells.

Suitably, in a device of the present invention, the transfer means (iii) may transfer an amount (such as a pre-determined amount) of a cell population directly from a patient to said chamber (i.e. without the need for the storage container) and/or the removal means (xi) may remove a population of cells, comprising undifferentiated cells, from the chamber directly into a patient (i.e. without the need for the storage container)..

Suitably, the transfer means (iii) may comprise a peristaltic pump, which pump transfers a cell population from a storage container via interconnecting means, tubing for instance, to the chamber. Preferably, the interconnecting means passes through a coulter counter or other suitable cytometer (as mentioned in (ii)). In this way, the cell concentration of the cell population can be calculated during transfer of the cell population from the storage container to the chamber.

Preferably, the storage container(s) mentioned in (iii) and (xi) above are storage bags, which are advantageously disposable. Much by preference the storage container of (iii) is different to the storage container of (xi), the first being an input storage container the latter being an output storage container.

Preferably, the further transfer means (v) comprises a reservoir of agent in order to ensure that enough agent is available at any one time. When the transfer means is a syringe, the reservoir may be provided by a further syringe, such that as one syringe become depleted the other syringe commences to supply the agent.

Preferably, the carbon dioxide control means for controlling the concentration of carbon dioxide in the chamber comprises a valve, which allows the introduction of a pre-determined amount of carbon dioxide from a gas cylinder. The pre-determined amount of

carbon dioxide is calculated using the known volume of air in the chamber, tubing and output storage container and the measured volume of the cell population from the input storage container. Suitably, the carbon dioxide is introduced into the chamber through a port which is the same as that through which the agent is added, in this way the carbon dioxide can be used to blow through all remaining agent in the port and surrounding equipment. In addition to the above, the carbon dioxide control means may further comprise an inflatable air bladder to accommodate the extra volume of air that results from the introduction of additional carbon dioxide into the system. Alternatively, the input storage container, once empty, may act as an air bladder. Preferably, carbon dioxide is introduced to produce 5% final concentration in the chamber.

The temperature control means (vii) may suitably comprise heating means, which heating means may be provided by a heat plate positioned beneath the chamber for example. Alternatively, the heating means may be provided by a heated water jacket located adjacent the chamber or a portion thereof. Much by preference, the temperature in the chamber is controlled at between about 25°C to about 37°C.

The mixing means (viii) preferably comprises at least one paddle arm, which slowly rotates in order to mix the cell population and the agent in the chamber. A magnetic stirrer could alternatively be used. As a yet further alternative, the chamber could be agitated mechanically, for example by gentle shaking. An advantage of using a mixing means comprising at least one paddle arm is that the arm can also be designed to act as a scraper during the harvesting of the cells, i.e. such that as the arm slowly rotates the cells attached to the surface of the chamber are gently dislodged. Advantageously, the mixing means is operable to effect gyratory motion.

Preferably, the removal means (xi) comprises a peristaltic pump. Advantageously, the removal means draws the cell population, including undifferentiated cells, through an output port at the bottom of the chamber and into the output storage container via interconnecting means, such as tubing for instance.

Alternatively or in addition to the removal means, a free ion sequestering and/or chelating agent, for example an anticoagulant such as EDTA, may be added to the chamber prior to removal of a population of cells therefrom. The addition of such an ion sequestering and/or chelating agent to the chamber causes stem cell colonies to disaggregate into a single cell suspension and therefore facilitates cell removal from the chamber by flushing means.

The sealing means (xii) preferably comprises a heat sealer, which heat sealer closes the output storage container by pressing together two portions of the container or two portions of the interconnecting means immediately before the container until there is a complete seal. For example, the heat sealer may result in a seal that is about 2cm in width, the heater sealer then cuts the seal along a centre portion thereof.

Preferably, each portion of the device according to the present invention is independently controllable by a central computer system. Advantageously, the computer system is capable of receiving input signals from a portion of the device, effecting calculations based on those input signals and sending output signals to a same or further portion of the device.

The device of the present invention has several advantages, in particular the device ensures that a consistent result is obtained each time the procedure is carried out and that no wastage of valuable agent occurs. In addition, the device may be programmed to alert the user when retrodifferentiation has finished and/or to display the time remaining before completion. If programmed to do so, the device can prompt the user to purchase new agent. That is to say, the device may monitor the use of the agent and the amount stored, and thus may prompt the user when the amount of stored agent falls below a critical level.

The device of the present invention may further comprise a plurality of chambers each for producing an undifferentiated cell committed to different cell lineages, e.g. muscle, hair, neurones, etc. Thus, simultaneous production of undifferentiated cells committed to different cell lineages may occur. Preferably, when a plurality of chambers is used each chamber has a separate outlet port and a separate output storage container.



Cell lineage may be controlled in the device of the present invention by using differently treated plastics for the chamber(s) or by the addition of different chemicals into each chamber.

- 5 Preferably, one or more of the components of the device are disposable. For example, one or more of the following components may be disposable: the chamber, the input storage container, the output storage container, the interconnecting means connecting the storage containers with the chamber, and the further transfer means (v). Suitably, the disposable components may be supplied as a cartridge for insertion into the device. By
- 10 way of example only, a cartridge may comprise a first blood bag (input storage container), a chamber and a second (output) blood bag wherein the chamber is connected to each of the first and second blood bags by tubing (interconnecting means). Suitably, the device may be considered as a system being part instrument and part disposable.
- 15 Preferably, the chamber and/or other components of the device in contact with the cell population are formulated from USP Class VI material, polycarbonate plaques or other plastics not capable of causing other forms of transformants of cells.

20 Preferably, the cell population comprising committed cells is blood. Preferably, the agent is an antibody.

In a further aspect, the present invention provides a method of preparing an undifferentiated cell, the method comprising retrodifferentiating a more committed cell to an undifferentiated cell, wherein the retrodifferentiation of the more committed cell

25 occurs to a more committed cell in or from a buffy coat blood sample.

In a yet further aspect, the present invention provides a method of preparing an undifferentiated cell, the method comprising contacting a more committed cell in or from a buffy coat blood sample with an agent that causes the more committed cell to

30 retrodifferentiate into an undifferentiated cell.

In one preferred embodiment of the present invention, the more committed cell is not a cancer cell. In another preferred embodiment of the present invention, the agent is neither carcinogenic nor capable of promoting cancer growth.

- 5 Preferably the committed cells are differentiated. Preferably the committed cells are committed haematopoietic cells, such as cell selected from CFC-T cells, CFC-B cells, CFC-Eosin cells, CFC-Bas cells, CFC-GM cells, CFC-MEG cells, BFC-E cells, CFC-E cells, T cells and B cells, more preferably B cells.
- 10 Preferably the undifferentiated cells are undifferentiated cells selected from a pluripotent stem cell, a haematopoietic stem cell, a neuronal stem cell, an epithelial stem cell, a mesenchymal stem cell and an embryonic stem cell. Preferably, the undifferentiated cells are characterised by one or more of following cell surface marker designations: CD34<sup>+</sup>, HLA-DR<sup>-</sup>, CD38<sup>-</sup>, CD117, AC133, CD90 and/or CD45<sup>low</sup>. More preferably the
- 15 undifferentiated cell is CD34<sup>+</sup> and CD38<sup>-</sup>, even more preferably, CD34<sup>+</sup>, CD38<sup>-</sup>, HLA-DR<sup>-</sup> and CD45<sup>low</sup>.

Suitably, the undifferentiated cells are MHC class I<sup>+</sup> and/or MHC class II<sup>+</sup> cells.

- 20 Preferably, the agent engages a receptor that mediates capture, recognition or presentation of an antigen at the surface of the committed cells. More preferably, the receptor is an MHC class I antigen or an MHC class II antigen, such as a class I antigen selected from Human-Leukocyte-Associated (HLA)-A receptor, an HLA-B receptor, an HLA-C receptor, an HLA-E receptor, an HLA-F receptor or an HLA-G receptor or a class II
- 25 antigen selected from an HLA-DM receptor, an HLA-DP receptor, an HLA-DQ receptor or an HLA-DR receptor.

- Suitably, the receptor may comprise a  $\beta$ -chain having homologous regions. In one embodiment, the receptor may comprise at least the homologous regions of the  $\beta$ -chain of
- 30 HLA-DR.

In a preferred embodiment, the agent is an antibody to the receptor, such as a monoclonal antibody to the receptor. Specific examples include CR3/43 and monoclonal antibody TAL.1B5.

- 5 Suitably, the agent may modulate MHC gene expression, for example MHC class I<sup>+</sup> and/or MHC class II<sup>+</sup> expression.

In a further preferred embodiment of the present invention there is provided a method of increasing the relative number of cells having a cell surface marker designation  
10 CD34<sup>+</sup> and/or HLA-DR<sup>+</sup> and/or CD38<sup>+</sup> and/or CD117 and/or AC133 and/or CD90 and/or CD45<sup>low</sup> in a buffy coat blood sample, the method comprising contacting a more committed cell in a buffy coat blood sample with an agent that operably engages said committed cells, such as the relative number of CD34<sup>+</sup> and/or HLA-DR<sup>+</sup> and/or CD38<sup>+</sup> and/or CD117 and/or AC133 and/or CD90 and/or CD45<sup>low</sup> cells increases as a result of  
15 said engaging.

In a yet further aspect, the present invention provides a method of preparing an undifferentiated cell, the method comprising contacting one or more differentiated cells in a cell population with retrodifferentiation means effective to displace the ratio of normal  
20 differentiated cells in said population, whereby one or more of said differentiated cells is caused to retrodifferentiate to an undifferentiated cell(s).

In a further aspect, the present invention provides the use of retrodifferentiating means to displace the ratio of normal differentiated cells in a cell population to effect  
25 retrodifferentiation of one or more of said differentiated cells to an undifferentiated cell(s).

In a yet further aspect, the present invention provides a method of preparing an undifferentiated cell, the method comprising retrodifferentiating a differentiated cell in a  
30 cell population to an undifferentiated cell, wherein the environment comprising said cell population comprising one or more differentiated cells is changed from a first environment to a second environment wherein the free ion concentration of said second

environment is effectively modified as compared with the first environment so as to cause one or more of said differentiated cells to retrodifferentiate to an undifferentiated cell(s).

5 In a yet further aspect, the present invention provides a method of preparing an undifferentiated cell, the method comprising contacting one or more differentiated cells in a cell population with retrodifferentiation means effective to displace the ratio of normal differentiated cells, culturing the cell population in a ion free or ion sequestered first environment, and changing the first environment to a second environment wherein the concentration of ions present in the second environment is effectively modified as  
10 compared with the first environment, thus to effect one or more of the differentiated cells to retrodifferentiate to an undifferentiated cell(s).

Preferably, the retrodifferentiating means is any means which causes disruption of the ratio of normal differentiated cells in a cell population, which is hereinafter called  
15 negative selection, within the cell population and thus causes a disruption of the ratio of normal differentiated cells in a cell population.

The retrodifferentiating means may be, for example, any one or more of the following: an antibody (pure and conjugated (i.e. bound to fixed and free ligands such as magnetic,  
20 glass or polystyrene beads for example); Histopaque, LymphoPrep (Sigma) or any other density gradient medium used to separate cells according to density of the cells; or Dextran (which causes sedimentation of red blood cells for example). Other suitable means that cause cell displacement are shown in Vettese-Dadey (The Scientist, Sep 13 1999, 13 (18): 21).

25 Preferably, the free ion concentration of the second environment is increased as compared with that of the first environment.

More preferably, the relative free ion concentration of the first and second environments  
30 is increased, i.e. the concentration of free ions in the second environment is increased. Preferably, the relative ion concentration is increased sufficiently to cause one or more of the differentiated cells to retrodifferentiate to an undifferentiated cell(s). By way of

example only, transfer of cells from a medium containing 5mM EDTA (a free ion sequestering and/or chelating agent) to a further medium containing less EDTA or no EDTA causes an increase in the free ion concentration (e.g. calcium ion concentration) sufficient to cause retrodifferentiation.

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Preferably, the free ion is an anion.

Preferably, the free ion is a group I or group II metal.

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Preferably, the anion is a calcium ion and/or a magnesium ion.

Suitably, the free ion concentration of an environment may be modified by treating the environment with an agent capable of relatively changing the free ion concentration of the environment.

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Thus, in a preferred embodiment the present invention provides a method of preparing an undifferentiated cell, the method comprising retrodifferentiating a differentiated cell in a cell population to an undifferentiated cell, wherein the environment comprising said cell population is modified by treating a first environment with an agent capable of relatively changing the free ion concentration of the environment to effect a second environment.

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For instance, the first environment may be treated with one or more free ion sequestering agents the presence of which is subsequently removed or reduced thus to effect a second environment having a relatively increased free ion concentration, thus effecting retrodifferentiation of one or more differentiated cells in the cell population. That is to say, the sequestering agent may be removed or reduced for instance by physically/chemically removing the or some of the sequestering agent from the first environment or by transferring the cell population to a second environment without such a free ion sequestering agent or with a free ion sequestering agent at a lower concentration than in the first environment.

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In any event, the second environment has an increased free ion concentration as compared with the first environment, thus effecting retrodifferentiation of the one or more differentiated cells in the cell population.

- 5 Alternatively, the cell population may be cultured in a first environment comprising a low or zero concentration of free ions followed by transferring the cell population to or adjusting the first environment so that it becomes a second environment comprising ions or comprising ions at a higher concentration than the first environment, thus effecting retrodifferentiation of one or more differentiated cells in the cell population. The term
- 10 "low" used herein includes an environment with a relatively low free ion starting concentration relative to the final concentration of the second environment.

- Thus, in a preferred embodiment the present invention also provides a method of preparing an undifferentiated cell, the method comprising retrodifferentiating a
- 15 differentiated cell in a cell population to an undifferentiated cell, wherein the environment comprising said cell population comprising one or more differentiated cells is changed from a first environment having a low or zero concentration of free calcium or magnesium ions to a second environment comprising free calcium or magnesium ion or comprising free calcium or magnesium ions at a higher concentration than the first
- 20 environment, thus effecting retrodifferentiation of one or more differentiated cells in the cell population.

- Without wishing to be bound by theory, it is thought that changes in the ion concentration, in particular increases in the ion concentration, causes cells in the cell
- 25 population to aggregate into colonies (for instance, undergo homotypic aggregation), and that the physical contact between such cells may induce them to retrodifferentiate.

Preferably, the sequestering agent is an ion chelating agent.

- 30 Preferably, the sequestering agent comprises both an amine and a carboxylic group.

Preferably, the sequestering agent comprises a plurality of  $-N(CH_2CO_2H)_n$  groups, wherein  $n=1$  or  $n=2$ .

Suitably, the sequestering agent may be selected from any one or more of the following:

- 5 EDTA, heparin, EGTA, DTPA, trisodium citrate and other similar chelating agents and/or anticoagulants.

Suitably, the sequestering agent should be added in a sufficiently high concentration such that removal of the presence thereof causes retrodifferentiation. Typically, the  
10 concentration of the sequestering agent sufficient to cause retrodifferentiation when the presence thereof is removed is more than or equal to about 2mM.

In one preferred embodiment of the present invention, the more committed cell is not a cancer cell. In another preferred embodiment of the present invention, the agent is neither  
15 carcinogenic nor capable of promoting cancer growth.

Preferably the committed cells are differentiated. Preferably the committed cells are committed haematopoietic cells, such as cell selected from CFC-T cells, CFC-B cells, CFC-Eosin cells, CFC-Bas cells, CFC-GM cells, CFC-MEG cells, BFC-E cells, CFC-E  
20 cells, T cells and B cells, more preferably B cells.

Preferably the undifferentiated cells are undifferentiated cells selected from a pluripotent stem cell, a haematopoietic stem cell, a neuronal stem cell, an epithelial stem cell, a mesenchymal stem cell and an embryonic stem cell. Preferably, the undifferentiated cells  
25 are characterised by one or more of following cell surface marker designations:  $CD34^+$ ,  $HLA-DR^-$ ,  $CD38^-$ ,  $CD117$ ,  $AC133$ ,  $CD90$  and/or  $CD45^{low}$ . More preferably the undifferentiated cell is  $CD34^+$  and  $CD38^-$ , even more preferably,  $CD34^+$ ,  $CD38^-$ ,  $HLA-DR^-$  and  $CD45^{low}$ .

30 Thus, in a further preferred embodiment the present invention also provides a method of preparing an undifferentiated cell, the method comprising retrodifferentiating a differentiated haematopoietic cell in a cell population to an undifferentiated cell, wherein

the environment comprising said cell population comprising one or more differentiated haematopoietic cells is changed from a first environment to a second environment wherein the free ion concentration of said second environment is effectively modified as compared with the first environment to cause one or more of said differentiated  
5 haematopoietic cells to retrodifferentiate to an undifferentiated cell(s).

Suitably, the undifferentiated cells are MHC class I<sup>+</sup> and/or MHC class II<sup>+</sup> cells.

In one embodiment, the cell population including committed cells is a buffy coat blood  
10 sample or is from a buffy coat blood sample.

Advantageously, a method according to the present invention may be used to effectively culture erythroid progenitors for the production of erythrocytes, which erythrocytes may be used to replenish shortages in blood supplies for example. Suitably, a method  
15 according to the present invention may be used to produce megakaryocytes for use in platelet production.

In order that the present invention may be clearly understood and readily carried into effect reference will now be made, by way of example, to the accompanying drawings.  
20

Figure 1 shows a perspective view of a device in accordance with the present invention with the front panel thereof open, together with an insert showing a section of the device removed from its surroundings;

25 Figure 2 shows a perspective view of the device of Figure 1 with the front panel thereof closed;

Figure 3 shows a photograph of blood cells;

30 Figure 4 shows a chart of the differentiation of LSCs to form T cells and B cells;



Figure 5 shows a chart of the differentiation of MSCs to form eosinophils, basophils, neutrophils, megakaryocytes, monocytes, erythrocytes, granulocytes, mast cells, NKs, and lymphocytes;

5 Figure 6 shows lymphohaematopoietic progenitor cells;

Figure 7 shows the appearance of CD45<sup>+</sup>CD14<sup>+</sup> cells after treatment with CR3/43 antibodies;

10 Figures 8 shows a microscope picture of differentiated B cells;

Figure 9 shows a microscope picture of undifferentiated cells formed by retrodifferentiation of B cells;

15 Figure 10 shows a microscope picture of the same undifferentiated cells as Figure 9 but at  
a lower magnification;

Figure 11 shows a microscope picture of differentiated B cells;

20 Figure 12 shows a microscope picture of undifferentiated cells formed by the  
retrodifferentiation of B cells:

Figure 13 shows a microscope picture of the formation of differentiated granulocyte cells from the same undifferentiated cells of Figure 12;

25

Figure 14 shows at two different magnifications, microscope pictures of an untreated blood sample from a BCLL patient;

Figure 15 shows microscope pictures of a treated blood sample;

30

Figures 16 and 17 show time-lapse microscope pictures during the treatment of blood samples;

Figure 18 shows a Southern blot;

Figure 19 shows further Southern blots obtained using peripheral blood cells from patients with B-CLL;

5

Figure 20 shows the use of three agents to increase the relative number of CD34<sup>+</sup> cells in a cell population;

Figure 21 shows a colony assay of stem cells using inverted bright field microscopy;

10

Figure 22 shows an adherent cell layer when viewed with inverted bright field microscopy; and

Figure 23 shows two still images from a time lapse video during treatment of B cells with CR3/43 mab.

15

## Detailed description of the invention

### I. Device

20

The device is generally depicted by reference number 1 and comprises a housing 2 having a front panel 3, which panel 3 can be opened to allow access to the inside of the device 1. An inlet storage container, namely a blood bag 4, hangs from a support hook 5. The support hook 5 forms part of an electronic balance (not shown), which is operable to weigh the input blood bag 4.

25

The device further comprises a chamber 6, which chamber 6 is interconnected to the input blood bag 4 via tubing 7. The tubing 7 comprises a transparent window 8, which window is positioned inside a coulter counter flow cell 9. A peristaltic pump (not shown) is operable to draw the blood from the input blood bag 4 through the coulter counter flow cell 9 and into the chamber 6.

30

Syringes 10 and 11 contain antigen and are each driven by a stepper-motor (not shown). The syringes 10, 11 are permanently maintained at 4°C within a lockable insulated Peltier “refrigerator” 12. Two syringes 10, 11 are provided in order to ensure that the device  
 5 always has sufficient antibody supply. The sterility of the syringes 10, 11 is maintained by both antibiotics and disposable 0.2 µm filters (generally designated as reference numeral 13).

The device further comprises a carbon dioxide inlet port 14, which directs the carbon  
 10 dioxide from a gas cylinder (not shown) into the chamber 6 via tubing 15. The syringes 10, 11 are also in fluid communication with the chamber 6 via the tubing 15. A valve 16 controls the flow of carbon dioxide and antigen through the tubing 15. Heating means (not shown) is provided beneath the chamber 6. Within the chamber 6 there is a rotatable  
 15 paddle 17, which is operable to mix antibody and blood within the chamber 6. A timing device (not shown) monitors a period of incubation when the antibody and blood remain within the chamber 6. The time remaining for incubation may be displayed on the control panel display 18.

An outlet storage container, namely an outlet blood bag 19 is also hung in the device 1  
 20 and is interconnected with the chamber 6 via tubing 20. The tubing 20 passes through a heat sealer 21, which is operable to seal the tube 20 and thus the outlet blood bag 19. A further peristaltic pump (not shown) is provided to pump the contents of the chamber 6 into the outlet blood bag 19.

25 The chamber 6 together with the tubing 7, 20 and the output blood bag 19 are disposable items which are disposed of after each procedure.

In operation, the user inserts into the device 1 an output blood bag 19, a chamber 6 and tubing 7, 20. The tubing 20 is passed through the heat sealer 21 and the peristaltic pump.  
 30 The tubing 7 is passed through the other peristaltic pump and the coulter counter flow cell 9. The user also inserts blood bag 4 containing blood from a patient and attaches it to the tubing 7. In addition to the above the user attaches the tubing 15 of the chamber 6 to the

sterile filter 13. The user then closes the front panel 3 of the housing 2 and starts the device 1 using the device's keypad 22.

The device 1 automatically weighs the input blood bag 4. The weight of the input blood bag 4 is sent automatically to a central computing system (not shown) located within the device 1. From the weight of the input blood bag 4 the computing system can determine the blood volume in the bag 4. The peristaltic pump then draws blood from the input blood bag 4 through the tubing 7. The blood flows through the transparent window 8 of the tubing 7 and the coulter counter 9 determines the cell concentration passing through the tubing 7. The coulter counter sends a signal direct to the central computing system. The central computing system determines from the calculated volume of blood and the cell concentration the correct volume of antibody which needs to be syringed into the chamber 6 by one or more of the syringes 10, 11. The peristaltic pump continues to pump the blood from the bag 4 until a signal is received from the central computing system, which stops the pump. The signal from the central computing system is sent in response to a signal from the coulter counter 9 to the central computing system, which signal is sent when the coulter counter 9 senses that no further cells are passing the window 8.

The central computing system then signals the stepper motor (not shown) attached to syringes 10, 11 to compress one or more of the syringes 10, 11 to deliver the calculated volume of antibody into chamber 6 via tubing 15.

The device then, optionally, introduces carbon dioxide through the carbon dioxide inlet port 14 by opening the valve 16 (the open and close mechanism of valve 16 being controllable by the central computer system). The amount of carbon dioxide to be added is calculated by the central computing system based on the known volume of air in the chamber 6, tubing 7, 20 and the output blood bag 19 and the calculated volume of blood from the input blood bag. The final concentration of carbon dioxide in the chamber 6 is brought to about 5%. The carbon dioxide is introduced into the chamber 6 through the tubing 15. The carbon dioxide thus blows through any remaining antibody in the tubing 15, thus ensuring that all of the released antibody is added to chamber 6. The input blood

bag 4 once empty acts as an air bladder to accommodate the additional gas volume following introduction of the carbon dioxide.

5 The device may further comprise a heater (not shown), under the control of the central computer system, which heater is situated beneath the chamber 6 and controls the temperature in the chamber 6 to between 25 and 37°C. A thermostat connected to the heater prevents over- or under-heating.

10 The blood and antibody agent are then incubated in the chamber 6 for a pre-determined period. Suitably, the incubation period does not exceed 24 hours and is preferably between 4 to 8 hours. The time selected should be sufficient to allow the retrodifferentiation reaction take place.

15 During incubation the paddle arms 17 slowly rotate to effect mixing of the antibody and the blood.

20 Upon completion of the incubation period, the paddle arms 17 continue to rotate and effectively act as scraper arms to dislodge any cells attached to the surface of the chamber 6 and thus to facilitate harvesting of the cells. The peristaltic pump draws the contents of the chamber 6 (i.e. blood containing undifferentiated cells, i.e. stem cells) from the bottom of the chamber 6 and into the output blood bag 19. The pump continues pumping until a measured volume of blood (as determined by the use of a calibrated peristaltic pump) has entered the output blood bag 19.

25 Finally, the heat sealer 21 clamps the tubing 20 and causes a length of about 2cm of the tubing 20 to be sealed, the sealer 21 then cuts the tubing 20 approximately centrally of the seal.

30 The device 1 may then notify the user that the process is complete.

The user can then remove the output blood bag 19. The chamber 6 together with the tubing 7, 20 and the input blood bag 4 may be disposed of.

The output blood bag 19 can, if necessary, be transferred to a purification system, for example to a system for identifying cells having cell surface markers characteristic of undifferentiated cells, although morphological changes may also be used as a guide. The purification system may be used to remove antibody agent and/or optionally enrich the undifferentiated (stem) cells. A suitable purification system is the CliniMACs/Isolex purification system.

## II. Undifferentiated cells and differentiated cells

There are many undifferentiated cells and differentiated cells found *in vivo* and the general art is replete with general teachings on them.

By way of example, with respect to cells of the haematopoietic cell lineages, reference may be made to *inter alia* Levitt and Mertelsman 1995 (Haematopoietic Stem Cells, published by Marcel Dekker Inc - especially pages 45-59) and Roitt *et al.* (Immunology, 4th Edition, Eds. Roitt, Brostoff and Male 1996, Publ. Mosby - especially Chapter 10).

An undifferentiated cell is an immature cell that does not display a mature differentiated character but is capable of yielding progeny that do. A well-known example of an undifferentiated cell is a stem cell.

Stem cells are undifferentiated immature cells, capable of self-renewal (division without limit) and differentiation (specialization). These juvenile cells are abundant in a developing embryo; however, their numbers decrease as development progresses. By contrast, an adult organism contains a limited number of stem cells which are confined to certain body compartments.

It is generally believed that stem cells are either monopotent, bipotent or pluripotent. Monopotent and bipotent stem cells are more restricted in development and give rise to one or two types of specialized cells, respectively. In contrast, the pluripotent stem

cells (PSCs) can differentiate into many different types of cells, giving rise to tissue (which constitute organs) or in the case of totipotent stem cells, the whole organism.

Pluripotent stem cells, unlike monopotent or bipotent, are capable of multilineage differentiation, giving rise to a tissue which would consist of a collection of cells of different types or lineages.

The Haematopoietic Stem Cell is an example of a pluripotent stem cell which is found among marrow cells and gives rise to all the various blood cells (including leukocytes and erythrocytes).

Blood is a fluid tissue, consisting of Lymphocyte (Ly), Monocytes (Mo), Neutrophils (Ne), Basophils (Ba), Eosinophils (Eso), Platelets (Pl) and Red Blood Cells (Rbc) – see Figure 3. This specialized tissue is produced by the differentiation of Haematopoietic Stem Cells (Hsc). In general, the white blood cells (inside larger circle) fight infections while red blood cells (inside smaller circle) transport nutrients, oxygen and waste products around the body.

Previously, haematopoietic stem cells were extracted by isolation from (i) bone marrow, (ii) growth factor mobilised peripheral blood or (iii) cord blood (placenta). Recently, haematopoietic stem cells have been prepared from embryonic stem (ES) cells, which are extracted from embryos obtained using *in vitro* fertilization techniques. These undifferentiated cells are capable of multi-lineage differentiation and reconstitution of all body tissue i.e. are totipotent.

The above mentioned extraction methods are cumbersome, sometime hazardous and in certain instances can be argued unethical, especially, in the case of the embryonic stem cells extraction method.

There are a number of undifferentiated stem cells of the haematopoietic lineage. These include pluripotent stem cells (PSCs), lymphoid stem cells (LSCs) and myeloid stem cells (MSCs), known collectively as lymphohaematopoietic progenitor cells (LPCs).

LSCs and MSCs are each formed by the differentiation of PSCs. Hence, LSCs and MSCs are more committed than PSCs.

5 Examples of differentiated cells of the haematopoietic lineage include T cells, B cells, eosinophils, basophils, neutrophils, megakaryocytes, monocytes, erythrocytes, granulocytes, mast cells, and lymphocytes.

10 T cells and B cells are formed by the differentiation of LSCs. Hence, T cells and B cells are more committed than LSCs. In more detail, the chain of differentiation is LSC -> pro-B-cell or prothymocyte. Pro-B-cell -> pre-B-cell -> mature B-cell -> plasma cell. Prothymocyte -> common thymocyte -> mature thymocytes (helper/inducer or cytotoxic/suppressor lineages) – see Figure 4.

15 Eosinophils, basophils, neutrophils, megakaryocytes, monocytes, erythrocytes, granulocytes, mast cells, NKs, and lymphocytes are formed by the differentiation of MSCs. Hence, each of these cells are more committed than MSCs. In more detail, the chain of differentiation is MSC -> immature megakaryoblast (-> megakaryoblast -> megakaryocyte -> platelet) or proerythroblast (-> erythroblast -> reticulocyte -> erythrocyte) or myelomonocytic stem cell, a bipotent stem cell that differentiates to either  
20 a myeloblast (-> promyelocyt -> myelocyt -> granulocyte) or a monoblast (-> promonocyte -> monocyte -> macrophage) – see Figure 5.

25 The pathways of differentiation of haemotopoiesis have thus been extensively characterised and the various cell stages are readily identifiable according to morphology and lineage-specific cell surface markers (see below).

30 Other stem cells include neural stem cells, multipotent stem cells that can generate neurons, astrocytes and oligodendrocytes (Nakafuku and Nakamura, 1995, J. Neurosci Res., vol 41(2): 153-68; Anderson, 1994, FASEB J., vol 8(10): 707-13; Morshead et al., 1994, Neuron, Vol 13(5): 1071-82). Skeletal muscle satellite cells are another type of stem cell, more specifically a distinct class of myogenic cells that are maintained as quiescent stem cells in the adult and can give rise to new muscle cells when needed



(Bischoff, 1986, Dev Biol., vol 115(1): 129-39). Other types of stem cells are epithelial stem cells, a subset of basal cells, endodermal stem cells and mesenchymal stem cells.

A very important type of stem cells is embryonic stem (ES) cells. These cells have been extensively studied and characterised. Indeed, ES cells are routinely used in the production of transgenic animals. ES cells have been shown to differentiate *in vitro* into several cell types including lymphoid precursors (Potocnik *et al.*, 1994, EMBO J., vol 13(22): 5274-83) and neural cells. US 5,843,780 and US 6,200,806 disclose the isolation of primate ES cells. ES cells are characterised by a number of stage-specific markers such as stage-specific embryonic markers 3 and 4 (SSEA-3 and SSEA-4), high molecular weight glycoproteins TRA-1-60 and TRA-1-81 and alkaline phosphatase (Andrews *et al.*, 1984, Hybridoma, vol 3: 347-361; Kannagi *et al.*, 1983, EMBO J., vol 2: 2355-2361; Fox *et al.*, 1984, Dev. Biol., vol 103: 263-266; Ozawa *et al.*, 1985, Cell. Differ., vol 16: 169-173).

Various antigens are associated with undifferentiated and differentiated cells. The term "associated" here means the cells expressing or capable of expressing, or presenting or capable of being induced to present, or comprising, the respective antigen(s).

Most undifferentiated cells and differentiated cells comprise Major Histocompatibility Complex (MHC) Class I antigens and/or Class II antigens. If these antigens are associated with those cells then they are called Class I<sup>+</sup> and/or Class II<sup>+</sup> cells.

Each specific antigen associated with an undifferentiated cell or a differentiated cell can act as a marker. Hence, different types of cells can be distinguished from each other on the basis of their associated particular antigen(s) or on the basis of a particular combination of associated antigens.

Examples of these marker antigens include the antigens CD34, CD19 and CD3. If these antigens are present then these particular cells are called CD34<sup>+</sup>, CD19<sup>+</sup> and CD3<sup>+</sup> cells respectively. If these antigens are not present then these cells are called CD34<sup>-</sup>, CD19<sup>-</sup> and CD3<sup>-</sup> cells respectively.

In more detail, PSCs are CD34<sup>+</sup> DR<sup>-</sup> TdT<sup>-</sup> cells (other useful markers being CD38<sup>-</sup> and CD36<sup>+</sup>). LSCs are DR<sup>+</sup>, CD34<sup>+</sup> and TdT<sup>+</sup> cells (also CD38<sup>+</sup>). MSCs are CD34<sup>+</sup>, DR<sup>+</sup>, CD13<sup>+</sup>, CD33<sup>+</sup>, CD7<sup>+</sup> and TdT<sup>+</sup> cells. B cells are CD19<sup>+</sup>, CD21<sup>+</sup>, CD22<sup>+</sup> and DR<sup>+</sup> cells.

- 5 T cells are CD2<sup>+</sup>, CD3<sup>+</sup>, and either CD4<sup>+</sup> or CD8<sup>+</sup> cells. Immature lymphocytes are CD4<sup>+</sup> and CD8<sup>+</sup> cells. Activated T cells are DR<sup>+</sup> cells. Natural killer cells (NKs) are CD56<sup>+</sup> and CD16<sup>+</sup> cells. T lymphocytes are CD7<sup>+</sup> cells. Leukocytes are CD45<sup>+</sup> cells. Granulocytes are CD13<sup>+</sup> and CD33<sup>+</sup> cells. Monocyte macrophage cells are CD14<sup>+</sup> and DR<sup>+</sup> cells. Additional details are provided in Figures 4 and 5.

10

Embryonic stem cells express SSEA-3 and SSEA-4, high molecular weight glycoproteins TRA-1-60 and TRA-1-81 and alkaline phosphatase. They also do not express SSEA-1, the presence of which is an indicator of differentiation. Other markers are known for other types of stem cells, such as Nestin for neuroepithelial stem cells (J. Neurosci, 1985, Vol 5: 3310). Mesenchymal stem cells are positive for SH2, SH3, CD29, CD44, CD71, CD90, CD106, CD120a and CD124, for example, and negative for CD34, CD45 and CD14.

15

- Alternatively, or in addition, many cells can be identified by morphological characteristics. The identification of cells using microscopy, optionally with staining techniques is an extremely well developed branch of science termed histology and the relevant skills are widely possessed in the art. Clearly staining of cells will only be carried out on aliquots of cells to confirm identity since stains in general cause cell death.

20

- 25 Hence, by looking for the presence of the above-listed antigen markers it is possible to identify certain cell types (e.g. whether or not a cell is an undifferentiated cell or a differentiated cell) and the specialisation of that cell type (e.g. whether that cell is a T cell or a B cell).

30

Undifferentiated cells may comprise any components that are concerned with antigen presentation, capture or recognition. Preferably, the undifferentiated cell is an MHC Class I<sup>+</sup> and/or an MHC Class II<sup>+</sup> cell.

The more committed cell may comprise any components that are concerned with antigen presentation, capture or recognition. Preferably, the more committed cell is an MHC Class I<sup>+</sup> and/or an MHC Class II<sup>+</sup> cell.

5

The more committed cell is any cell derived or derivable from an undifferentiated cell. Thus, in one preferred embodiment, the more committed cell is also an undifferentiated cell. By way of example therefore the more committed undifferentiated cell can be a lymphoid stem cell or a myeloid stem cell, and the undifferentiated cell is a pluripotent stem cell.

10

In another preferred embodiment, the more committed cell is a differentiated cell, such as a CFC-T cell, a CFC-B cell, a CFC-Eosin cell, a CFC-Bas cell, a CFC-Bas cell, a CFC-GM cell, a CFC-MEG cell, a BFC-E cell, a CFC-E cell, a T cell, a B cell, an eosinophil, a basophil, a neutrophil, a monocyte, a megakaryocyte or an erythrocyte; and the undifferentiated cell is a myeloid stem cell, a lymphoid stem cell or a pluripotent stem cell.

15

If the more committed cell is a differentiated cell then preferably the differentiated cell is a B lymphocyte (activated or non-activated), a T lymphocyte (activated or non-activated), a cell from the macrophage monocyte lineage, a nucleated cell capable of expressing class I or class II antigens, a cell that can be induced to express class I or class II antigens or an enucleated cell (i.e. a cell that does not contain a nucleus - such as a red blood cell).

20

In alternative preferred embodiments, the differentiated cell is selected from any one of a group of cells comprising large granular lymphocytes, null lymphocytes and natural killer cells, each expressing the CD56 and/or CD16 cell surface receptors.

25

The differentiated cell may even be formed by the nucleation of an enucleated cell.

30

### III. Agents

The agent operably engages the more committed cell in order to retrodifferentiate that cell into an undifferentiated cell. In this regard, the agent for the retrodifferentiation of the more committed cell into the undifferentiated cell may act in direct engagement or in indirect engagement with the more committed cell.

The agent may act intracellularly within the more committed cell. However, preferably, the agent acts extracellularly of the more committed cell.

An example of direct engagement is when the more committed cell has at least one cell surface receptor on its cell surface, such as a  $\beta$ -chain having homologous regions (regions that are commonly found having the same or a similar sequence) such as those that may be found on B cells, and wherein the agent directly engages the cell surface receptor.

Another example, is when the more committed cell has a cell surface receptor on its cell surface such as an  $\alpha$ -chain having homologous regions such as those that may be found on T cells, and wherein the agent directly engages the cell surface receptor.

An example of indirect engagement is when the more committed cell has at least two cell surface receptors on its cell surface and engagement of the agent with one of the receptors affects the other receptor which then induces retrodifferentiation of the more committed cell.

The agent for the retrodifferentiation of the more committed cell into an undifferentiated cell may be a chemical compound or composition. Preferably, however, the agent is capable of engaging a cell surface receptor on the surface of the more committed cell. Thus, in a preferred embodiment, the agent operably engages a receptor present on the surface of the more committed cell - which receptor may be expressed by the more committed cell, such as a receptor that is capable of being expressed by the more committed cell.

For example, preferred agents include any one or more of cyclic adenosine monophosphate (cAMP), a CD4 molecule, a CD8 molecule, a part or all of a T-cell receptor, a ligand (fixed or free), a peptide, a T-cell receptor (TCR), an antibody, a cross-reactive antibody, a monoclonal antibody, or a polyclonal antibody. Growth factors may  
 5 also be used, such as haematopoietic growth factors, for example erythropoietin and granulocyte-monocyte colony stimulating factor (GM-CSF).

If the agent is an antibody, a cross-reactive antibody, a monoclonal antibody, or a polyclonal antibody, then preferably the agent is any one or more of an antibody, a cross-reactive antibody, a monoclonal antibody, or a polyclonal antibody to any one or more of:  
 10 the  $\beta$  chain of a MHC class II antigen, the  $\beta$  chain of a MHC HLA-DR antigen, the  $\alpha$  chain of a MHC class I or class II antigen, the  $\alpha$  chain of HLA-DR antigen, the  $\alpha$  and the  $\beta$  chain of MHC class II antigen or of a MHC class I antigen. An example of a suitable antibody is CR3/43 (supplied by Dako).

15 The term "antibody" includes the various fragments (whether derived by proteolytic cleavage or recombinant technology) and derivatives that retain binding activity, such as Fab,  $F(ab')_2$  and scFv antibodies, as well as mimetics or bioisosteres thereof. Also included as antibodies are genetically engineered variants where some of the amino acid  
 20 sequences have been modified, for example by replacement of amino acid residues to enhance binding or, where the antibodies have been made in a different species to the organism whose cells it is desired to treat according to the methods of the invention, to decrease the possibility of adverse immune reactions (an example of this is 'humanised' mouse monoclonal antibodies).

25 Agents used to effect the conversion of a more committed cell to an undifferentiated cell preferably act extracellularly of the more committed cell. In particular, it is preferred that the more committed cell comprises a receptor that is operably engageable by the agent and the agent operably engages the receptor.

30 For example the receptor may be a cell surface receptor. Specific examples of cell surface receptors include MHC class I and class II receptors. Preferably, the receptor

comprises an  $\alpha$ - component and/or a  $\beta$ - component, as is the case for MHC class I and class II receptors.

More preferably, the receptor comprises a  $\beta$ -chain having homologous regions, for example at least the homologous regions of the  $\beta$ -chain of HLA-DR.

Alternatively, or in addition, the receptor comprises an  $\alpha$ -chain having homologous regions, for example at least the homologous regions of the  $\alpha$ -chain of HLA-DR.

10 Preferably, the receptor is a Class I or a Class II antigen of the major histocompatibility complex (MHC). In preferred embodiments the cell surface receptor is any one of: an HLA-DR receptor, a DM receptor, a DP receptor, a DQ receptor, an HLA-A receptor, an HLA-B receptor, an HLA-C receptor, an HLA-E receptor, an HLA-F receptor, or an HLA-G receptor. In more preferred embodiments the cell surface receptor is an HLA-DR receptor.

15 Preferably, the agent is an antibody to the receptor, more preferably the agent is a monoclonal antibody to the receptor.

20 Another preferred example of an agent is one that modulates MHC gene expression such as MHC Class I<sup>+</sup> and/or MHC Class II<sup>+</sup> expression.

In a preferred embodiment, the agent is used in conjunction with a biological response modifier. Examples of biological response modifiers include an alkylating agent, an immunomodulator, a growth factor, a cytokine, a cell surface receptor, a hormone, a nucleic acid, a nucleotide sequence, an antigen or a peptide. A preferred alkylating agent is or comprises cyclophosphamide.

Other preferred biological response modifiers include compounds capable of upregulating MHC class I and/or class II antigen expression. In a preferred embodiment, this is so as to allow an agent that binds to an MHC receptor to work more effectively.

Since any cell type can be made to express MHC class I and/or class II antigens, this should provide a method for retrodifferentiation a wide variety of cell types whether they constitutively express class I and/or class II MHC antigens or not.

#### 5 IV. Methods for retrodifferentiating cells

In the methods of the invention, a population of cells comprising committed cells is contacted with an agent that operably engages one or more committed cells in the population. The cell population is then incubated so as to allow those cells that have been  
10 operably engaged by the agent to progress through the retrodifferentiation process and ultimately become undifferentiated.

Preferably the contacting step comprises the agent engaging with any one or more of the following: homologous regions of the  $\alpha$ -chain of class I antigens, homologous regions of  
15 the  $\alpha$ -chain of class II antigens, a CD4 cell surface receptor, a CD8 cell surface receptor, homologous regions of the  $\beta$ -chain of class II antigens in the presence of lymphocytes, homologous regions of the  $\alpha$ -chain of class I antigens in the presence of lymphocytes, homologous regions of the  $\alpha$ -chain of class II antigens in the presence of lymphocytes. Preferably the contacting step occurs in the presence of the biological response modifier  
20 (see above).

Typically, the population of cells is derived from a biological sample, such as blood or related tissues including bone marrow, neuronal tissue from the central nervous system or peripheral nervous system, muscle tissue, or epidermis and/or dermis tissue from skin  
25 (i.e. by way of oral scraping for instance). Preferably biological material is of post-natal origin. It is preferred to use whole blood or processed products thereof, such as plasma or the buffy coat, since their removal from subjects can be carried out with the minimum of medical supervision. Blood samples are typically treated with anticoagulents such as heparin or citrate. Cells in the biological sample may be treated to enrich certain cell  
30 types, remove certain cell types or dissociate cells from a tissue mass. Useful methods for purifying and separating cells include centrifugation (such as density gradient

centrifugation), flow cytometry and affinity chromatography (such as the use of magnetic beads comprising monoclonal antibodies to cell surface markers or panning) (see Vettese-Dadey *The Scientist* (Sep. 13 1999) 13 (18): 21). By way of example, Ficoll-Hypaque separation is useful for removing erythrocytes and granulocytes to leave mononuclear  
 5 cells such as lymphocytes and monocytes.

Since the cells are essentially primary cultures, it may necessary to supplement populations of cells with suitable nutrients to maintain viability. Suitable culture conditions are known by the skilled person in the art. Nonetheless, treatment of cell  
 10 populations is preferably initiated as soon as possible after removal of biological samples from patients, typically within 12 hours, preferably within 2 to 4 hours. Cell viability can be checked using well known techniques such as trypan blue exclusion.

Cell populations are generally incubated with an agent for at least two hours, typically  
 15 between 2 and 24 hours, preferably between 2 and 12 hours. Incubations are typically performed at from about room temperature, for example about 22°C, up to about 37°C, including 33°C. The progress of the retrodifferentiation procedure can be checked periodically by removing a small aliquot of the sample and examining cells using microscopy and/or flow cytometry. Alternatively, the device can comprise tracking  
 20 means for on-line monitoring the progress of the retrodifferentiation procedure.

Once the relative numbers of the desired cell type have increased to a suitable level, which may for example be as low as 0.1% or as high as 5%, the resulting altered cell populations may be used in a number of ways. With respect to the numbers of  
 25 undifferentiated cells formed, it is important to appreciate the proliferative ability of stem cells. Although under some circumstance, the numbers of stem cells or other undifferentiated cells formed may appear to be low, studies have shown that only 50 pluripotent haematopoietic stem cells can reconstitute an entire haematopoietic system in a donor mouse. Thus therapeutic utility does not require the formation of a large number  
 30 of cells.



Conversion of more committed cells to undifferentiated cells may also be carried out *in vivo* by administration of the agent, admixed with a pharmaceutically carrier or diluent, to a patient. However it is preferred in many cases that retrodifferentiation is performed *in vitro/ex vivo*.

5

Treated populations of cells obtained *in vitro* may be used subsequently with minimal processing. For example they may be simply combined with a pharmaceutically acceptable carrier or diluent and administered to a patient in need of stem cells.

10 It may however be desirable to enrich the cell population for the undifferentiated cells or purify the cells from the cell population. This can conveniently be performed using a number of methods (see Vattese-Dadey - The Scientist 13 (18): 21 Sep 1999). A device according to the present invention may optionally comprise purification or isolation means for enriching said undifferentiated cells or recovering said undifferentiated cells  
15 from the altered cell population. For example cells may be purified on the basis of cell surface markers using chromatography and/or flow cytometry. Nonetheless, it will often be neither necessary nor desirable to extensively purify undifferentiated cells from the cell population since other cells present in the population (for example stromal cells) may maintain stem cell viability and function.

20

Flow cytometry is a well-established, reliable and powerful technique for characterising cells within mixed populations as well as for sorting cells. Thus, the purification or isolation means may comprise a flow cytometer. Flow cytometry operates on the basis of physical characteristics of particles in liquid suspension, which  
25 can be distinguished when interrogated with a beam of light. Such particles may of course be cells. Physical characteristics include cell size and structure or, as has become very popular in recent years, cell surface markers bound by monoclonal antibodies conjugated to fluorescent molecules.

30 Kreisseg *et al.*, 1994, J. Hematother 3(4): 263-89, state, "Because of the availability of anti-CD34 monoclonal antibodies, multiparameter flow cytometry has become the tool of choice for determination of haemopoietic stem and progenitor cells" and goes on to

describe general techniques for quantitation and characterisation of CD34-expressing cells by flow cytometry. Further, Korblyng *et al.*, 1994, Bone Marrow Transplant. 13: 649-54, teaches purification of CD34<sup>+</sup> cells by immunoadsorption followed by flow cytometry based on HLA-DR expression. As discussed above, CD34<sup>+</sup> is a useful  
 5 marker in connection with stem cells/progenitor cells.

Flow cytometry techniques for sorting stem cells based on other physical characteristics are also available. For example, Visser *et al.*, 1980, Blood Cells 6:391-407 teach that stem cells may be isolated on the basis of their size and degree of  
 10 structuredness. Grogan *et al.*, 1980, Blood Cells, 6: 625-44 also teach that "viable stem cells may be sorted from simple haemopoietic tissues in high and verifiable purity".

As well as selecting for cells on the basis of the presence of a cell surface marker or  
 15 other physical property (positive selection), cell populations may be enriched, purified using negative criteria. For example, cells that possess lineage specific markers such as CD4, CD8, CD42 and CD3 may be removed from the cell population by flow cytometry or affinity chromatography.

20 A very useful technique for purifying cells involves the use of antibodies or other affinity ligands linked to magnetic beads. The beads are incubated with the cell population and cells that have a cell surface marker, such as CD34, to which the affinity ligand binds are captured. The sample tube containing the cells is placed in a magnetic sample concentrator where the beads are attracted to the sides of the tube.  
 25 After one or more wash stages, the cells of interest have been partially or substantially completely purified from other cells. When used in a negative selection format, instead of washing cells bound to the beads by discarding the liquid phase, the liquid phase is kept and consequently, the cells bound to the beads are effectively removed from the cell population.

30

These affinity ligand-based purification methods can be used with any cell type for which suitable markers have been characterised or may be characterised.

Urbankova *et al.*, 1996. (J. Chromatogr B Biomed Appl. 687: 449-52) teaches the micropreparation of haematopoietic stem cells from a mouse bone marrow suspension by gravitational field-flow fractionation. Urbankova *et al.*, 1996, further comments  
 5 that the method was used for the characterisation of stem cells from mouse bone marrow because these cells are bigger than the other cells in bone marrow and it is therefore possible to separate them from the mixture. Thus physical parameters other than cell surface markers may be used to purify/enrich for stem cells.

- 10 Cell populations comprising undifferentiated cells and purified undifferentiated cells produced by the methods of the invention may be maintained *in vitro* using known techniques. Typically, minimal growth media such as Hanks, RPMI 1640, Dulbecco's Minimal Essential Media (DMEM) or Iscove's Modified Dulbecco Medium are used, supplemented with mammalian serum such as FBS, and optionally  
 15 autologous plasma, to provide a suitable growth environment for the cells. In a preferred embodiment, stem cells are cultured on feeder layers such as layers of stromal cells (see Deryugina *et al.*, 1993, Crit Rev. Immunology, vol 13: 115-150). Stromal cells are believed to secrete factors that maintain progenitor cells in an undifferentiated state. A long term culture system for stem cells is described by  
 20 Dexter *et al.*, 1977 (J. Cell Physiol, vol 91: 335) and Dexter *et al.*, 1979 (Acta. Haematol., vol 62: 299).

For instance, Lebkowski *et al.*, 1992 (Transplantation 53(5): 1011-9) teaches that human CD34<sup>+</sup> haematopoietic cells can be purified using a technology based on the  
 25 use of monoclonal antibodies that are covalently immobilised on polystyrene surfaces and that the CD34<sup>+</sup> cells purified by this process can be maintained with greater than 85% viability. Lebkowski *et al.*, 1993 (J. Hematother, 2(3): 339-42) also teaches how to isolate and culture human CD34<sup>+</sup> cells. See also Haylock *et al.*, 1994 (Immunomethods, vol 5(3): 217-25) for a review of various methods.

30

Confirmation of stem cell identity can be performed using a number of *in vitro* assays such as CFC assays (see also, the examples). Very primitive haematopoietic stem cells

are often measured using the long-term culture initiating cell (LTC-IC) assay (Eaves *et al*, 1991, J. Tiss. Cult. Meth. Vol 13: 55-62). LTC-ICs sustain haemopoiesis for 5 to 12 weeks.

- 5 Cell populations comprising undifferentiated cells and purified preparations comprising undifferentiated cells may be frozen for future use. Suitable techniques for freezing cells and subsequently reviving them are known in the art. A device according to the present invention may optionally further comprise freezing means for freezing cell populations comprising undifferentiated cells and/or purified preparations  
10 comprising undifferentiated cells.

In one aspect, preferably the retrodifferentiation occurs to cells from or in buffy coat blood samples. The term "buffy coat" means the layer of white cells that forms between the layer of red cells and the plasma when unclotted blood is centrifuged or  
15 allowed to stand.

#### V. Displacement of the ratio of normal differentiated cells in a cell population

In normal tissue the relative number of the various types of cells, including  
20 differentiated and undifferentiated cells, at a given time is usually constant. For example, a white blood cell count of a healthy 21 year old individual would typically be about  $8 \times 10^6$  per ml, of which 27% is lymphocytes, 6% is monocytes and 67% is granulocytes. The level of such cells (including both the relative number and the absolute cell count) is disturbed during disease, such is the case in leukaemic blood of  
25 patients with B cell choronic lymphocytic leukaemia (B-CLL).

The relative number (i.e. ratio) of differentiated cells can be perturbed, disturbed or displaced for example in mononuclear cell fractions by layering whole blood or buffy coats on histopaque to remove granulocytes, thus to effect retrodifferentiation of  
30 differentiated cells to undifferentiated cells, which undifferentiated cells will self renew (proliferate) and redifferentiate into a variety of cell types to replenish the displaced cells.

The relative number (i.e. ratio) of differentiated cells can also be perturbed, disturbed or displaced in, for example, buffy coats (obtained from healthy blood donors) following removal (by centrifugation on density gradient medium or negative selection using antibody coated magnetic beads) of red blood cells, platelet, granulocytes, monocytes and T lymphocytes. This treatment may be called negative selection or enrichment of a certain type of differentiated cell and is an example of a displaced tissue. When such cells are cultured, for example, in a calcium containing medium such as Iscove's Modified Dulbeccos Medium (ISDM), differentiated cells will undergo retrodifferentiation to undifferentiated cells, which undifferentiated cells will self renew (proliferate) and redifferentiate into a variety of cell types to replenish the displaced cells.

During this process cells are first seen to cluster into colonies (undergo homocytic aggregation) in order to undergo retrodifferentiation. Once the more committed cells convert into undifferentiated cells they acquire the ability to proliferate and develop into a variety of redifferentiated cell types, in an effort to replenish the relative number of displaced cells.

Retrodifferentiating means operable to displace the ratio of normal differentiated cells in a cell population include, for example, antibodies (pure and conjugated, i.e. bound to fixed and free ligands such as magnetic, glass or polystyrene beads for instance); Histopaque, LymphoPrep or any density gradient medium used to separate cells according to the density of the cells; or Dextran (capable of causing sedimentation of red blood cells for instance). Other suitable retrodifferentiating means may be identified in a paper by Vettese-Dadey (see The Scientist (Sep. 13 1999) 13 (18): 21).

Exposure of displaced cells to suitable chelating agent, including EDTA, EGTA and heparin, (which chelating agent may be referred to as a biological response modifier) may cause even more committed cells to retrodifferentiate into undifferentiated cells. For example, exposure of displaced cells to EDTA for a pre-determined period of time followed by culturing the cells in a calcium containing medium containing cortisone

caused more committed cells to retrodifferentiate into undifferentiated cells. These cells in turn self renewed and embarked on a new differentiation pathway, giving rise to erythroid progenitors such as burst forming unit-erythroid (BFU-erythroid). The erythroid progenitors can be cultured and expanded for long periods of time and may be used in the treatment red blood disorder and red blood shortages.

#### **VI. Changing the free ion concentration of a medium comprising a cell population**

Exposure of differentiated cells to an ion chelating agent, such as for example EGTA, for a given period of time followed by culturing of such cells in a calcium containing medium, such as IMDM, containing hydrocortisone causes more committed cells to retrodifferentiate into undifferentiated cells. These cells in turn self renew and embark on a new differentiation pathway giving rise to megakaryocytic progenitors, such as colony forming unit-megakaryocytes (CFU-Meg), which ultimately give rise to platelets.

#### **VII. Methods for recommitting undifferentiated cells**

One important application of undifferentiated cells of the present invention is in the reconstitution of tissues, for example nervous tissue or haematopoietic cells. This involves differentiating the undifferentiated cells produced by the methods of the invention. This may be carried out by simply administering the undifferentiated cells to a patient, typically at a specific site of interest such as the bone marrow, spinal cord or lung, and allowing the natural physiological conditions within the patient to effect differentiation. A specific example of this is the reconstitution or supplementation of the haematopoietic system, for example in the case of AIDS patients with reduced number of CD4<sup>+</sup> lymphocytes.

Alternatively, differentiation (also termed "recommitting", herein) can be effected *in vitro* and expanded cells then, for example, administered therapeutically. This is generally performed by administering growth factors. For example, retinoic acid has been used to differentiate ES cells into neuronal cells. Methylcellulose followed by

co-culture with a bone marrow stromal line and IL-7 has been used to differentiate ES cells into lymphocyte precursors (Nisitani *et al.*, 1994, *Int. Immuno.*, vol 6(6 ): 909-916). Le Page (New Scientist 16 December 2000) teaches that ES cells can be differentiated into lung epithelial cells. Bischoff, 1986 (*Dev. Biol.*, vol 115(1): 129-39) teaches how to differentiate muscle satellite cells into mature muscle fibres. Neural precursor cells can be expanded with basic fibroblast growth factor and epidermal growth factor (Nakafuku and Nakamura, 1995, *J. Neurosci. Res.*, vol 41(2): 153-168). Haematopoietic stem cells can be expanded using a number of growth factors including GM-CSF, erythropoietin, stem cell factor and interleukins (IL-1, IL-3, IL-6) – see Metcalf, 1989 (*Nature*, vol 339: 27-30) for a review of these various factors.

Potocnik *et al.*, 1994 (*EMBO J.*, vol 13(22): 5274-83) even demonstrated the differentiation of ES cells to haematopoietic cells using low oxygen (5%) conditions.

Thus, in a preferred embodiment of the present invention the undifferentiated cell is then committed into a recommitted cell, such as a differentiated cell. The recommitted cell may be of the same lineage to the more committed cell from which the undifferentiated cell was derived. Alternatively, the recommitted cell may be of a different lineage to the more committed cell from which the undifferentiated cell was derived. For example, a B lymphocyte may be retrodifferentiated to a CD34<sup>+</sup> CD38<sup>-</sup> HLA-DR<sup>-</sup> stem cell. The stem cell may be subsequently recommitted along a B cell lineage (the same lineage) or a lymphoid lineage (different lineage).

Commitment of the undifferentiated cell into a recommitted cell, such as a differentiated cell, can be effected in various way known to the skilled person. Notably by culturing the undifferentiated cells in a particular manner and in a particular media differentiation into selected cells can be effected.

By way of example only, undifferentiated cells can be differentiated into cardiac myocytes by following the underlined culturing regime:

Day 1: Pass the undifferentiated cells at normal density on a gelatinised plate to free the culture of contaminating fibroblast cells.

5 Trypsinize the cells as for normal passage until the colonies lift off. Handle gently in order to maintain the loosely connected clumps of cells together. Then directly plate the cells 1:3 into bacterial grade Petri dishes in LIF free ES cell culture medium (see below).

Day 3: Aspirate the medium carefully. Avoid sucking up too many of the aggregates. Then add new medium.

10

Day 5: Aspirate as in Day 3 and replace the medium.

Day 7: Plate the cells into a 24 well tissue culture grade plate.

15 Day 9: Change half of the medium and observe beating.

Day 11: Change half of the medium and observe beating.

20 By further way of example, the undifferentiated cells can be differentiated into glial cells and neuron by following the underlined procedure:

Day 1: Pass the undifferentiated cells at normal density on a gelatinised plate to free the culture of contaminating fibroblast cells.

25 Trypsinize the cells as for normal passage until the colonies lift off. Handle gently in order to maintain the loosely connected clumps of cells together. Then directly plate the cells 1:3 into bacterial grade Petri dishes in LIF free medium containing 1 $\mu$ M all-trans retinoic acid (available from Sigma).

30 Day 3: Collect cell aggregates and re-plate in tissue culture dishes (approximately 25 cell aggregates per 6 cm tissue culture dish) in ES cell culture medium without LIF or RA. Aspirate the medium carefully.



Day 8: Change half of the medium. From this day on at least 10% of the cells exhibit neuronal phenotypes. They are specifically stained with Cresyl Violet and strongly positive for the N-CAM antigen.

5

A skilled person would be readily aware of suitable procedures for effecting the commitment of an undifferentiated cell into any differentiated cell selected.

10

An undifferentiated stem cell of the present invention may be cultured using any routine embryonic stem (ES) cell culturing technique. By way of example only, a suitable media for undifferentiated or ES cell culture is detailed below

To prepare 100ml of medium:

15

DMEM (GIBCO cat# 11965-062	80ml
15% FCS	15ml
Pen/Strep	1ml
L-Glutamine	1ml
MEM non essential amino acids (GIBCO cat#11140-050	1ml
Lif ( $10^3$ U/ml)	1ml
BME (0.1 M)	0.2ml

The Lif comes in 1ml ampules as LIF ESGRO AMRAD at  $10^7$  U/ml, this can be diluted in 100 ml DMEM and 10% FCS and stored in 5ml aliquotes at - 20.

20 With regard to BME, 0.1 ml of BME (14.4M) can be added to 14.3ml PBS, filtered through a 0.2 micron acrodisc, and stored at - 20 for up to 1 month.

A further suitable medium may be for example PMEF media a 100ml of which is prepared as detailed below:

DMEM (GIBCO cat#11965-062)	88ml
10%FCS	10ml
Pen/Strep	1ml
L-Glutamine	1ml
BME (0.1 M)	0.2 ml

- 5 Other suitable media for culturing ES cells would be readily apparent to those skilled in the art.

#### VIII. Assays for identifying retrodifferentiating agents

- 10 In addition to the agents mentioned above, further suitable agents may be identified using assay methods of WO96/23870 or of the present invention. In respect of the latter aspect, the present invention also provides a method for identifying a substance capable of retrodifferentiating a committed/differentiated cell to an undifferentiated cell, which method comprises contacting a population of cells comprising committed cells with a
- 15 candidate substance and determining whether there is an increase in the relative numbers of undifferentiated cells in said cell population, wherein said contacting occurs in the presence of a buffy coat.

- Suitable candidate substances include ligands that bind to cell surface receptors such
- 20 as antibody products (for example, monoclonal and polyclonal antibodies, single chain antibodies, chimeric antibodies and CDR-grafted antibodies), such as antibodies that bind to cell surface receptors. Cell surface receptors of particular interest are described above and include MHC receptors and surface proteins with CD designations, such as CD4 and CD8. Other ligands that bind to cell surface receptors
- 25 include growth factors.

Furthermore, combinatorial libraries, peptide and peptide mimetics, defined chemical entities, oligonucleotides, and natural product libraries may be screened for activity as retrodifferentiation agents. The candidate substances may be used in an initial screen in batches of, for example 10 substances per reaction, and the substances of those  
5 batches which show inhibition tested individually.

A typical assay comprises placing an aliquot of cells comprising committed cells in a suitable vessel such as a multiwell plate. A candidate substance is added to the well and the cells incubated in the well. Incubations are typically performed at from about room  
10 temperature, for example about 22°C, up to about 37°C. including 33°C.

Retrodifferentiation may be measured by removing a small aliquot of cells and examining the cells by microscopy and/or flow cytometry to determine whether there has been a change in the numbers of undifferentiated cells. Typically, the determination of changes  
15 in the numbers of undifferentiated cells is performed by monitoring changes in the numbers of cell having cell surface markers characteristic of undifferentiated cells, although morphological changes may also be used as a guide. Examples of suitable cell surface markers include CD34<sup>+</sup>. Alternatively, or in addition, decreases in the numbers of cells having cell surface markers typical of differentiated cells and not undifferentiated  
20 cells may be monitored, for example a reduction in the relative numbers of cells possessing lineage specific markers such as CD3, CD4 and CD8

Preferably, any increase in the numbers of cells having characteristics typical of undifferentiated cells occurs within 24 hours, preferably 4 to 8 hours, such that any  
25 changes cannot be solely accounted for by cell proliferation.

It may be desirable to prescreen for agents that bind to, for example, cell surface receptors, such as MHC class I or class II receptors. Any agents identified as binding to target cell surface receptors may then be used in the above assay to determine their effect  
30 on retrodifferentiation. As a particular example, phage display libraries which express antibody binding domains may be used to identify antibody fragments (typically scFvs) that bind to a target cell surface marker, such as the homologous region of the  $\beta$ -chain of

MHC class II receptors. Suitable binding assays are known in the art, as is the generation and screening of phage display libraries. Assays may also be used to identify optimised antibodies or antibody fragments, for example to screen a mutagenised library of derivatives of an antibody already shown to effect retrodifferentiation.

5

## IX. Uses

The present invention provides methods of and a device for retrodifferentiating committed cells to undifferentiated cells. In particular, the present invention provides a method and device for preparing a stem cell from a more differentiated cell. The clinical implications of this are enormous since stem cells are being used in a wide variety of therapeutic applications but up until now were difficult, cumbersome and sometimes ethically controversial to obtain.

15 Stem cells produced according to the present invention may be used to repopulate specific cell populations in a patient, such as a haematopoietic cell population or a subpopulation thereof, such as CD4 T-lymphocytes. The more committed cells used to produce the stem cells may be from the same patient or a matched donor. Thus stem cells produced according to the present invention may be used to heal and reconstitute specialised cell tissue and organs. For example, undifferentiated cells could be used to produce recommitted cells such as the cells lining the alveoli of the lungs, thus creating a mechanism by which damaged or diseased lung tissue can be replaced or repaired (see Le Page, New Scientist 19 December 2000, p 20).

25 Thus, the stem cells produced according to the present invention may be introduced into the patient to repopulate cells in the body. Suitably, the stem cells first graft and then repopulate.

Suitably, the stem cells may be introduced *in vivo* using liposomal transfer.

30

Thus, the present invention also encompasses a medicament comprising an undifferentiated cell prepared by any one of these processes or by the device admixed with a suitable diluent, carrier or excipient.

- 5 In one embodiment, the medicament comprising the undifferentiated cell may be used to produce a beneficial more committed cell, such as one having a correct genomic structure, in order to alleviate any symptoms or conditions brought on by or associated with a more committed cell having an incorrect genomic structure. Thus, the present invention also provides a process of removing an acquired mutation from a more
- 10 committed cell wherein the method comprises forming an undifferentiated cell by the method according to the present invention, committing the undifferentiated cell into a recommitted cell, whereby arrangement or rearrangement of the genome and/or nucleus of the cell causes the mutation to be removed.
- 15 Preferably the gene is inserted into the immunoglobulin region or TCR region of the genome.

- The present invention also provides a method of treating a patient suffering from a disease or a disorder resulting from a defective cell or an unwanted cell, the method
- 20 comprising preparing an undifferentiated cell by contacting a more committed cell with an agent that causes the more committed cell to retrodifferentiate into the undifferentiated cell, and then optionally committing the undifferentiated cell into a recommitted cell; wherein the undifferentiated cell, or the recommitted cell, affects the defective cell or the unwanted cell to alleviate the symptoms of the disease or disorder or to cure the patient of
- 25 the disease or condition.

- Alternatively, the undifferentiated cell could be used to produce a more committed cell that produces an entity that cures any symptoms or conditions brought on by or associated with a more committed cell having an incorrect genomic structure.
- 30

For example, the present invention may be used to prepare antibodies or T cell receptors to an antigen that is expressed by the more committed cell which has retrodifferentiated

into the undifferentiated cell. In this regard, the antigen may be a fetospecific antigen or a cross-reactive fetospecific antigen.

5 The present invention also includes a process of and a device for controlling the levels of undifferentiated cells and more committed cells. For example, the present invention includes a method comprising forming an undifferentiated cell by the method according to the present invention and then activating an apoptosis gene to affect the undifferentiated cell, such as bring about the death thereof.

10 In a preferred embodiment the present invention relates to a process of introducing a gene into the genome of an undifferentiated cell, wherein the process comprises introducing the gene into a more committed cell, and then preparing an undifferentiated cell by the method according to the present invention, whereby the gene is present in the undifferentiated cell.

15 In a more preferred embodiment the present invention relates to a process of introducing a gene into the genome of an undifferentiated cell, wherein the process comprises inserting the gene into the genome of a more committed cell, and then preparing an undifferentiated cell by the method according to the present invention, whereby the gene  
20 is present in the undifferentiated cell.

The gene may be a gene that renders the undifferentiated cell and more differentiated cells obtained therefrom more resistant to pathogenic infections such as a viral infection. In particular, by way of example, B lymphocytes from AIDS patients may be used to  
25 produce stem cells that are then engineered to be resistant to HIV infection. When expanded and introduced into the patients, the resulting helper T lymphocytes may also be resistant to HIV infection.

30 In an alternative embodiment the present invention relates to a process of introducing a gene into an undifferentiated cell, wherein the process comprises inserting the gene into the genome of a more committed cell, and then preparing an undifferentiated cell by the

method according to the present invention, whereby the gene is present in the genome of the undifferentiated cell.

In addition, the present invention also encompasses the method of the present invention  
5 for preparing an undifferentiated cell, wherein the method includes committing the undifferentiated cell into a recommitted cell and then fusing the recommitted cell to a myeloma. This allows the expression *in vitro* of large amounts of the desired product, such as an antibody or an antigen or a hormone etc.

10 The present invention encompasses an undifferentiated cell prepared by any one of these processes of the present invention.

In a further embodiment, the device and/or method according to the present invention may be used to effectively culture erythroid progenitors for the production of  
15 erythrocytes, which erythrocytes may be used to replenish shortages in blood supplies for example. Suitably, a method according to the present invention may be used to produce megakaryocytes for use in platelet production.

In an alternative embodiment, the device and/or method of the present invention may be  
20 used in the preparation of banks of undifferentiated cells, i.e. banks of treated cells.

Other aspects of the present invention include:

The use of any one of the agents of the present invention for preparing an undifferentiated  
25 cell from a more committed cell.

The use of an undifferentiated cell produced according to the method of the present invention for producing any one of a monoclonal or a polyclonal or a specific antibody from a B-lymphocyte or a T-lymphocyte; a cell from the macrophage monocyte lineage;  
30 a nucleated cell capable of expressing class I or class II antigens; a cell capable of being induced to express class I or class II antigens; an enucleated cell; a fragmented cell; or an apoptic cell.

The use of an undifferentiated cell produced according to the method of the present invention for producing effector T-lymphocytes from B-lymphocytes and/or *vice versa*.

- 5 The use of an undifferentiated cell produced according to the method of the present invention for producing any one or more of: a medicament, such as a medicament comprising or made from a B-lymphocyte, a T-lymphocyte, a cell from the macrophage monocyte lineage, a nucleated cell capable of expressing a class I or a class II antigen, a cell capable of being induced to express a class I or a class II antigen, or an enucleated  
10 cell.

The present invention also encompasses processes utilising the afore-mentioned uses and products or compositions prepared from such processes.

- 15 The present invention also encompasses a medicament comprising an undifferentiated cell according to the present invention or a product obtained therefrom admixed with a suitable diluent, carrier or excipient.

- In one preferred embodiment the medicament comprises an antibody or antigen obtained  
20 from an undifferentiated cell according to the present invention admixed with a suitable diluent, carrier or excipient.

- Preferably the medicament is for the treatment of any one of: cancer, autoimmune diseases, blood disorders, cellular or tissue regeneration, organ regeneration, the  
25 treatment of organ or tissue transplants, or congenital metabolic disorders.

- The methods of the invention and products obtained by those methods, such as undifferentiated cells, may be used in research, for example to study retrodifferentiation, differentiation and identify and study new developmental antigens and cluster  
30 differentiation antigens.



## X. Administration

Stem cells and recommitted cells of the present invention, as well as agents shown to retrodifferentiate cells, may be used in therapeutic methods. Preferably the cells or agents  
5 of the invention are combined with various components to produce compositions of the invention. More preferably the compositions are combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition (which may be for human or animal use). Suitable carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition of the invention  
10 may be administered by direct injection. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular, oral or transdermal administration.

Compositions comprising cells are typically delivered by injection or implantation.  
15 Cells may be delivered in suspension or embedded in a support matrix such as natural and/or synthetic biodegradable matrices. Natural matrices include collagen matrices. Synthetic biodegradable matrices include polyanhydrides and polylactic acid. These matrices provide support for fragile cells *in vivo* and are preferred for non-haemopoietic cells.

20 Delivery may also be by controlled delivery i.e. over a period of time which may be from several minutes to several hours or days. Delivery may be systemic (for example by intravenous injection) or directed to a particular site of interest.

25 Cells are typically administered in doses of from  $1 \times 10^5$  to  $1 \times 10^7$  cells per kg. For example a 70 kg patient may be administered  $14 \times 10^6$  CD34<sup>+</sup> cells for reconstitution of haematopoietic tissues.

The routes of administration and dosages described are intended only as a guide since  
30 a skilled practitioner will be able to determine readily the optimum route of administration and dosage for any particular patient and condition.

All of the methods detailed below are suitable for use with a device according to the present invention.

## A. MATERIALS AND METHODS

5

### **PATIENTS**

10 Blood samples were obtained in lavender top tubes containing EDTA from patients with B-cell chronic lymphocytic leukaemias, patients with antibody deficiency (including IgA deficiency and X-linked infantile hypogammaglobulinaemias), patients with HIV infections and AIDS syndrome, a patient with CMV infection, a patient with Hodgkin's lymphomas, a patient with acute T-cell leukaemia, a 6-days old baby with blastocytosis, various patients with various infections and clinical conditions, cord blood, bone marrow's, and enriched B-lymphocyte preparations of healthy blood donors.  
15 Additionally, buffy coat blood samples were obtained from healthy donors.

### **CLINICAL AND EXPERIMENTAL CONDITIONS**

20 The clinical and experimental treatment conditions of patients, including various types of treatment applied to their blood samples, are described in Table 1. Differential white blood cell (WBC) counts were obtained using a Coulter Counter and these are included in the same Table.

### **TREATMENT OF BLOOD**

25

Blood samples, once obtained, were introduced in a chamber together with pure monoclonal antibody to the homologous region of the  $\beta$ -chain of the HLA-DR antigen (DAKO) and left to mix at room temperature for a maximum of 24 hours. Some samples were mixed first for 15 minutes after which they were left to incubate in the chamber at  
30 22°C. The concentration of monoclonal antibody added to blood samples varied from 10-50  $\mu$ l/ml of blood.

In addition, other treatments were applied at the same concentrations and these included addition of a monoclonal antibody to the homologous region of the  $\alpha$ -chain of the HLA-DR antigen, a monoclonal antibody to the homologous region of class I antigens, a monoclonal antibody to CD4, a monoclonal antibody to CD8, and a PE conjugated monoclonal antibody to the homologous region of the  $\beta$ -chain of the HLA-DR antigen.

Other treatments included the simultaneous addition of monoclonal antibodies to the homologous regions of the  $\alpha$  and  $\beta$ -chains of the HLA-DR antigen to blood samples.

Furthermore, alkylating agents such as cyclophosphamide were added to blood samples in combination with pure monoclonal antibody to the homologous region of the  $\beta$ -chain of the HLA-DR antigen.

Following these treatments blood samples were stained with panels of labelled monoclonal antibodies as instructed by the manufacturer's instructions and then analysed using flow cytometry.

Incubation periods with monoclonal antibodies ranged from 2 hour, 4 hour, 6 hour, 12 hour to 24 hour intervals.

## **LABELLED ANTIBODIES**

The following monoclonal antibodies were used to detect the following markers on cells by flow cytometry: CD19 and CD3, CD4 and CD8, DR and CD3, CD56 & 16 and CD3, CD45 and CD14, CD8 and CD3, CD8 and CD28, simultest control (IgG1 FITC + IgG2a PE), CD34 and CD2, CD7 and CD13 & 33, CD10 and CD25, CD5 and CD10, CD5 and CD21, CD7 and CD5, CD13 and CD20, CD23 and CD57 and CD25 and CD45 RA (Becton & Dickenson and DAKO). Additional markers may include CD71 (red blood cell marker), CD61 (megakaryocyte marker), Glycophorin A (red blood cell marker), AC133 (stem cell marker), CD38 (primitive stem cell marker), CD90 (stem cell marker) and CD117 (pluripotent stem cell marker).

Each patient's blood sample, both treated and untreated, and including each buffy coat sample, was analysed using a plurality of the above panel in a device according to the present invention in order to account for the immunophenotypic changes that accompanied different types of treatments and these were carried out on different aliquots of the same blood sample. Untreated samples and other control treatments were stained and analysed simultaneously.

### FLOW CYTOMETRY

Whole blood sample was stained and lysed according to the manufacturer instructions. Flow cytometry analysis was performed on a FACScan@ with either simultest or PAINT A GATE software (BDIS) which included negative controls back tracking. 10,000 to 20,000 events were acquired and stored in list mode files.

### MORPHOLOGY

Morphology was analysed using microscopy and Wright's stain.

### PREPARING STEM CELLS FROM ENRICHED OR PURIFIED B-CLL (OR NORMAL) LYMPHOCYTES OR FROM BUFFY COAT BLOOD SAMPLES.

Aseptic techniques should be used throughout the following procedures:

#### (A) Mononuclear cell separation:

- (i) Obtain mononuclear cells from peripheral blood/buffy coat samples by centrifugation on Histopaque, Lymphoprep, or any Lymphocyte separation medium (sp. grav 1.077) for 30 mins at 400g.
- (ii) Collect mononuclear cells in a 50 ml conical tube and wash with 30 mls of Hank's balanced salt solution ( $\text{Ca}^{2+}$  and  $\text{Mg}^{+}$  free, Sigma) containing 2% heat-activated fetal calf serum (FCS) and 2 mM EDTA or 0.6% citrate, and centrifuge at 400g for 10 min.

(iii) After washing, count cells and assess viability using trypan blue and haemocytometer.

(iv) If B-cell count is high, above 70% ( $20 \times 10^9/L$ , WBC), proceed straight to A (vi).

5 (v) If B-cell count is low, below 70% ( $20 \times 10^9/L$ , WBC). Perform negative selection using Macs microbeads or FacsVantage purification technique, as described below in Section C.

(vi) Resuspend cell pellet at a concentration of  $3 \times 10^6/ml$  in IMDM medium (100  $\mu g/ml$  streptomycin), containing 10% FCS (heat inactivated) and 10% HS (heat inactivated), place in blood bag. Note: If no FCS and HS available, use 20% to 50% autologous plasma.

10

**PREPARING STEM (UNDIFFERENTIATED) CELLS IN MONONUCLEAR FRACTIONS OBTAINED FROM PERIPHERAL BLOOD SAMPLES OF PATIENTS WITH B-CLL.**

15

Follow the protocol for mononuclear cell separation (A) above, except that negative selection should not be performed when white blood cell count exceeds  $20 \times 10^6$  per ml of which more than 50% of the white blood cells are B cells.

20

The following procedures may be carried out in a device according to the present invention.

(B) Cell treatment using pure CR3/43 (Dako) monoclonal antibody:

25 After mononuclear cell separation has been achieved in A (vi), proceed with the following:

(i) Place input blood bag (from A(vi) above) on support hook,

(ii) Use a chamber with six wells, programme the device to add 2 mls of cell suspension from input blood bag [from A (vi) above] to each well of this multi-well culture tray or chamber.

30

(iii) Programme the device to treat an appropriate number of wells each with 99  $\mu\text{l/ml}$  (or 49.5  $\mu\text{l/ml}$  in respect of buffy coat samples) of CR3/43 (pure monoclonal antibody, Dako) from a syringe containing CR3/43 mab and leave the other wells untreated (negative control).

- 5 (iv) Incubate the chamber in 5%  $\text{CO}_2$  at  $37^\circ\text{C}$  (or  $33^\circ\text{C}$ ) in a humid atmosphere.

(C) Purification of Cells:

Various methods are known for the separation and purification of cells (see Vettese-  
10 Dadey Scientist 13(18):21 Sep 13 1999).

One suitable method is negative selection of B cells using MACS microbeads (Miltenyi Biotec, here it is best to follow manufacturer instructions):

- (i) Obtain mononuclear cells as in Section A above.
- 15 (ii) Pellet and resuspend cells in a final volume of 300  $\mu\text{l}$  per  $10^8$  total cells in HBSS (consisting of 2% FCS and 2 mM EDTA or 0.6% citrate).
- (iii) Add 100  $\mu\text{l}$  per  $10^8$  total cells of pure monoclonal antibody to CD2 (IgG1, DAKO).
- (iv) To the same cell suspension add 50 $\mu\text{l}$  per  $10^8$  per total cells of pure  
20 monoclonal antibody to CD33 (IgG1, DAKO).
- (v) Leave the mixture to incubate for 10 minutes at room temperature.
- (vi) Wash cells with HBSS (containing 2% FCS and 2 mM EDTA) and resuspend at a final concentration of 400  $\mu\text{l}$  per  $10^8$  total cells, with the same buffer. Centrifuge at 400g for 10 min. Resuspend pellet in HBSS (as above).
- 25 (vii) Add 100  $\mu\text{l}$  of rabbit anti-mouse IgG1 labelled microbeads per  $10^8$  total cells (or follow manufacturer instructions).
- (viii) Thoroughly mix cells and incubate at  $6^\circ\text{C}$  to  $12^\circ\text{C}$  (fridge) for 15 minutes.
- (ix) Again wash cells with HBSS (containing 2% FCS and 2mM EDTA),  
30 centrifuge at 400g x 10 mins and resuspend at a final concentration of 500 $\mu\text{l}$  per  $10^8$  total cells, with the same buffer.

(x) Assemble  $MS^+/RS^+$  column in the magnetic field of the MACS separator. If the mononuclear cell count is high use two  $MS^+/RS^+$  columns.

(xi) Wash column with 3 mls of HBSS (containing 2% FCS and 2 mM EDTA).

(xii) Pass cells through column and then wash with 4 x 500 $\mu$ l with HBSS  
5 (containing 2% FCS and 2mM EDTA).

(xiii) Elute and collect cells in a conical tube, then pellet and resuspended in IMDM and place in a blood bag as in Section A (vi).

#### D) FACSVantage purified B Cells:

10 (i) Obtain mononuclear cells from peripheral blood samples of B-CLL patients, as described in Section A, above.

(ii) Stain these cells with a combination of CD19-PE and CD20-FITC conjugated monoclonal antibodies to identify the B cells.

(iii) On the basis of CD19/CD20 fluorescence, sort approximately  $10^7$  cells using a  
15 Beckton Dickenson FACS Vantage and argon laser emitting at 488nm.

(iv) Wash purified cells with Hanks balanced salt solution containing 50% FCS and then allow to recover overnight at 37°C in a humidified incubator at 5% CO<sub>2</sub>.

(v) Pellet and resuspend cells, and place in a blood bag, as described in Section A

(vi) above and then treat with CR3/43 as described in Section B above.

20

### **PREPARING STEM CELLS IN WHOLE BLOOD CELLS**

Treatment of cells with pure CR3/43 (Dako) monoclonal antibody in whole blood may be carried out in a device according to the present invention:

25 (i) Select patients with WBC counts of 30-200 $\times 10^9$ /L (ranging from 73-95% B lymphocytes).

(ii) Collect blood by venipuncture into citrate, EDTA- or preservative free heparin containing tubes, place the blood in a blood bag. Preferably, collected blood is used immediately and, suitably, within about 6 hours from collection. However, blood  
30 stored/frozen in under nitrogen may also be used following defrosting.

(iii) Place the blood bag from (ii) comprising the whole blood in the device according to the present invention

(iv) Automated differential white blood cell count is conducted using a Coulter counter. Patients with white blood cell counts of  $30-200 \times 10^6$  per ml (of which 60-95% are B cells) are preferably selected.

(v) Viability of the blood may also be assessed automatically, suitable methods include either propidium iodide and flow cytometry or Trypan blue and a haemocytometer following lysis of red blood cells using ammonium chloride solution.

(vi) CR3/43 antibody is added to a sample of the whole blood in the chamber of the device, at a final concentration of  $1-3 \mu\text{l}/10^6$  cells (e.g. if WBC count was  $50 \times 10^9/\text{L}$  then  $50 \mu\text{l}$  of CR3/43 monoclonal antibody, mouse IgG concentration of  $159 \mu\text{g}/\text{ml}$ , should be added per ml of blood).

(vii) Mix blood and antibody thoroughly using a paddle in the chamber of the device and leave for a predetermined time period, not less than 2-hours, at room temperature, preferably between  $18^\circ\text{C}$  and  $37^\circ\text{C}$ , in the incubated chamber.

(viii) Analyse blood cells using flow cytometry, clonal assays, long term culture and/or PCT analysis 0 hr, 2 hr, 6 hr and 24 hr after the addition of mAbs using tracking means of the device.

Note: Due to the homotypic aggregation of B cells and the formation of adherent cells in the bottom of the test tube, induced by mAb CR3/43, thoroughly mix and sample cells using wide-bore pipette tips before analysis.

In order to obtain a uniform population of cells throughout the analysis, divide blood sample into separate aliquots in a plurality of chambers prior to CR3/43 treatment.

## 25 PREPARATION FOR ANALYSIS OF STEM CELLS PRODUCED BY TREATING CULTURED B CELLS WITH CR3/43 MONOCLONAL ANTIBODY.

Stem cells produced using the methods of the invention can be assessed at a number of times points, for example every 2hr, 7hr, 24hr, daily, 7 days or longer periods (months, following weekly feeding of cells with long term culture medium). One or more



treatment and control wells can be analysed at each time point, with remaining treatment and control wells being analysed at successive time period thereafter.

This assessment can also be made automatically by tracking means incorporated in the device of the present invention.

- (i) Gently remove non-adherent layer using a wide bore pipette and disrupt cell clumps by repeated aspiration through a wide bore pipette to obtain single cell suspension.
- 10 (ii) Using a cell scraper scrape adherent layer and disrupt gently cell clumps to obtain single cell suspension by repeated aspiration through a wide bore pipette.
- (iii) Alternatively, trypsinize adherent layer by first rinsing with HBSS and then adding 2 ml of 0.25% trypsin per well and incubate at 37C° for 10 minutes.
- (iv) Gently disturb cell clumps by repeated pipetting in a wide bore pipette.
- 15 (v) After 10 mins incubate with 20% of FCS to a final concentration to inactivate the trypsin.
- (vi) Culture cells obtained in (ii) or (v) above may be pooled for each type (i.e. treated and non-treated cells) at each time point and centrifuged at 400g for 10 mins.
- (vii) For PCR analysis (see C), the cell pellet (treated and non-treated cells) obtained
- 20 in (vi) should be used directly.
- (viii) For clonal assay analysis, the cell pellet (treated and non-treated cells) obtained in step (ii) or step (v) above are resuspended in IMDM containing 2% heat-activated FCS. A small aliquot of cells may be removed to count the cells and to assess their viability using trypan blue and a haemocytometer.
- 25 (ix) For FACs analysis, resuspend the cell pellet (treated and non-treated cells) obtained in step (ii) or step (v) above in Hanks Balanced Salt Solution (calcium and magnesium free) containing 2% heat activated FCS and 5mM EDTA.

#### ANALYSIS OF STEM CELLS:

The following methods can be used for the assessment of stem cells. The device according to the present invention may include tracking means for carrying out the or part of the methods hereinbelow detailed.

5 (A) Immunophenotype:

For Immunophenotypic analysis (using Flow Cytometry) for whole blood samples:

(i) Immunostain (according to manufacturer instructions), lyse the erythrocytes and wash the cells after the incubation period and treat with mAb. Lysing and wash solutions from Becton Dickinson may typically be used.

(ii) Leukocytes (in whole blood, mononuclear fraction, MACS microbeads negatively selected B cells or sorted B-CLL) should be labelled with mAbs conjugated directly to fluorescein isothiocyanate (FITC) or phycoerythrin (PE).

(iii) The panel markers used in the immunophenotypic analysis may be as follows:

- 15 • CD38 PE + CD45 FITC + CD34 PE-Cy5
- CD19 PE + CD10 FITC + CD34 PE-Cy5
- CD117 PE + CD3 FITC + CD34 PE-Cy5
- CD33 PE + CD61 FITC + CD34 PE-Cy5
- 20 • Glycophorine A PE + CD71 FITC + CD34 PE-Cy5
- AC133 PE + CD90 FITC + CD34 PE-Cy5
- CD19PE + CD5 FITC
- IgG1 PE + IgG1 FITC + IgG1 PE-Cy 5 (isotype negative control)

25 (iv) Double labelling using IMK+ kit (Becton Dickinson) may be performed: consisting of the following monoclonal antibody pairs:

- CD45-FITC and CD14-PE;
- CD19-PE and CD3-FITC;
- 30 • CD8-PE and CD4-FITC;
- HLA-DR-PE and CD3-FITC; and

- CD56, CD16-PE and CD3-FITC.

Also isotype match negative controls for IgG<sub>1</sub>-FITC and IgG<sub>2a</sub>-PE are included.

- 5 (v) The following additional antibodies can also be used which are manufactured by Dako and Becton Dickinson:

PE-conjugated: anti-CD8, anti-CD33, anti-13, anti-CD34, anti-CD19, anti-CD2, anti-CD14, anti-CD33 and anti-CD5;

10

FITC-conjugated: anti-CD3, anti-CD7, anti-IgM, anti-CD22, anti-CD20, anti-CD10, anti-CD7, anti-CD16, anti-TCR $\alpha\beta$ .

- (vi) The following can also be used.

- 15 • Also affinity purified IgG<sub>3</sub> mAb specific for CD34 (Dako) can be used and is detected with FITC- or PE-labelled goat anti-mouse immunoglobulin F(ab)<sub>2</sub> fragment as secondary antibody (DAKO).
- Quantum Red (PE - Cy5)-conjugated anti-CD34 (Dako) was also used.

- 20 (vii) Analyse cells using FACScan or FACS Vantage (Becton Dickinson) or any other flow cytometer. An equal number of events 100,000 cells should be analysed and the time noted.

- (viii) Analyse data using Proprietary Paint-a-Gate, Lysis II, Consort 30 and CellQuest software.

25

For Immunophenotypic analysis (using Flow Cytometry) of buffy coat samples for example:

- (i) Immunostain (according to manufacturer instructions), lyse the erythrocytes and wash the cells after the incubation period and treat with mAb. Lysing and wash solutions from Becton Dickinson may typically be used.
- 30 (ii) Leukocytes should be either doubly or singly labelled with mAbs conjugated directly to fluorescein isothiocyanate (FITC), phycoerythrin (PE) or RPE-Cy5.

The following labelled markers may suitably be used:

- CD34 (stem cell marker); CD19 (B lymphocyte marker); CD45 (leukocyte marker);  
 CD3 (T-lymphocyte marker); CD33 (myelocyte marker); CD71 (red blood cell  
 5 marker); CD61 (megakaryocyte marker); Glycophorin A (red blood cell marker);  
 AC133 (stem cell marker); CD38 (primitive stem cell marker); CD90 (stem cell  
 marker); CD10 (lymphoid stem cell marker); CD117 (pluripotent stem cell marker);  
 IgG1 (negative control).

10 (B) Morphology:

For morphological analysis:

Light Microscopy

- 15 (i) Resuspend cells thoroughly in IMDM containing 2% heat activated FCS using  
 wide-bore pipette tips.  
 (ii) Examine under a Leitz microscope using an appropriate staining solution, for  
 example May and Greenwald Staining Solution (BDH Chemical Ltd) may be used for the  
 analysis of myeloid and lymphoid cells. The skilled person will be readily aware of other  
 20 stains suitable for the identification of other colonies, for example Wright's or Giemsa  
 stains.  
 (iii) Morphological analysis of B-CLL lymphocytes can be performed in blood films  
 or cytocentrifuged preparations, respectively.
- 25 Confocal Microscopy  
 (i) Obtain B cells as described above (B-CLL or healthy B cells obtained from  
 buffy coat of healthy blood donors)  
 (ii) Treat B cells with CR3/43 monoclonal antibody as described above.  
 (iii) Add 2 ml of cell suspension to an organ culture dish (The bottom of this dish is  
 30 engineered to have a cover-slip).  
 (iv) Add 15 µl of monoclonal antibody to CD19 FITC-conjugate and 15µl of  
 monoclonal antibody to CD34 PE/Cy 5-conjugate (Quantum Red).

(v) Use Propidium Iodide to assess viability and Hoechst to stain the nuclei.

(C) PCR analysis of VDJ/JHF gene rearrangement

5 The VDJ and/or JHF region of the IgH gene was analysed by PCR (Perkin Elmer thermal cycler) using template DNA from B-CLL peripheral blood and buffy coat samples before and after (2hr, 6hr and 24hr) antibody treatment. This protocol is adapted from Stolc *et al* (American Journal of Hematology 38:1-8; 1991). The JHF primers are used for the positive detection of immunoglobulin heavy chain gene in a  
10 germ line configuration. The JH6 primers is used as a positive internal control for the immunoglobulin heavy chain gene. In B cells, leukaemic as well as in normal lymphocytes the JHF region is always deleted and therefore can not be amplified, while the JH6 gene an internal control, remain intact. The  $\beta$ -actin gene was used as a control.

15

(i) isolate genomic DNA from whole blood using Qiagen QiAmp Maxi blood kit. Use the maximum yield protocol. Extract all DNA from each sample (treated and untreated).

20

(ii) Run neat and 1:5 dilutions of DNA on a 1.5% agarose gel to assess the concentration and quality.

(iii) PCR - heat 2-3  $\mu$ L neat genomic DNA template at 96°C for 5 minutes

I) JH6a/JH6g JHFa/JHFg

25 Prepare a "mastermix" sufficient for 4 patients (8 reactions treated and untreated) in accordance with the table detailed below. This can be scaled up or down as appropriate.

	Volume/ $\mu$ L	[Final]
Nuclease-free water	370	--
PCR 10 x Buffer	80	1x
*25mM MgCl <sub>2</sub>	64	2mM
*5mM dNTPs	128	0.8mM
10 $\mu$ M P1	80	1 $\mu$ M
10 $\mu$ M P2	80	1 $\mu$ M
5U/ $\mu$ L Taq pol.	2	10 units

\*Vortex the tubes 2-3 times each for 5 seconds before use to equilibrate sample.

- 5 Aliquot 95  $\mu$ L of "mastermix" into each tube containing denatured genomic DNA. Set the thermocycler at 95°C for 1 min 30 secs; 55°C for 2 mins; 72°C for 1 mins 30 secs (35 cycles); and 72°C for 5 mins (1 cycle).

## II) $\beta$ -actin

10

Prepare a "mastermix" sufficient for 8 reactions as described in I) above. Scale up or down as appropriate.

	Volume/ $\mu$ L	[Final]
Nuclease-free water	380	--
PCR 10 x Buffer	80	1x
*25mM MgCl <sub>2</sub>	48	1.5mM
*5mM dNTPs	128	0.8mM
10 $\mu$ M P1	80	1 $\mu$ M
10 $\mu$ M P2	80	1 $\mu$ M
5U/ $\mu$ L Taq pol.	2	10 units

\*Vortex tubes 2-3 times each for 5 seconds before use to equilibrate sample.

15

Aliquot 95  $\mu$ L of "mastermix" into each tube containing denatured genomic DNA. Set the thermocycler at 95°C for 1 min 30 secs; 55°C for 2 mins; 72°C for 1 mins 30 secs (25 cycles); and 72°C for 5 mins (1 cycle).

## 5 Primers for PCR

The first set of primers, designed to amplify a 240 bp fragment of the IgH JHF gene were as follows:

JHFa ~~AAA GGT GCT GGG GGT CCC CTG~~  
 JHfb ~~CCC AGT GCT GGA AGT ATT CAG C~~

10

The second set of primers, designed to amplify a 242 bp fragment of the IgH JHF gene joining region 6 were as follows:

JH6a ~~CAT TGT GAT TAC TAC TAC TAC~~  
 JH6b ~~GAT CCT CAA GGC ACC CCA GTG C~~

15

The third set of primers, designed to amplify a 249 bp fragment of the  $\beta$ -actin gene were as follows:

Beta ACT-1 ~~AAG GCC AAC CGC GAG AAG AT~~  
 Beta ACT-2 ~~TCG GTG AGG ATC TTC ATG AG~~

20

## (D) Southern analysis of VDJ gene rearrangement

- (i) Digest the Genomic DNA from treated and untreated peripheral blood samples or purified B cells (from B-CLL patients), using *Bam*HI/*Hind*III – typically cells from a number of wells are required to give a sufficient amount of DNA to conduct the analysis.
- (ii) The digests were resolved on 0.8% agarose gels and transferred to GeneScreen® nylon membranes (Dupont) according to manufacturer's instructions (Southern, 1975).

25

(iii) The rearrangement of the IgH gene can be characterised by analysing the J region of the IgH locus, using  $^{32}\text{P}$ -labeled human  $\text{J}_\text{H}$  DNA probe isolated from placental genomic DNA (Calbiochem, Oncogene Science).

(iv) Autoradiographs should be kept at  $-70^\circ\text{C}$  for several days prior to developing.

5

(E) Long Term Culture:

Cell cultures prepared as described above can be maintained for longer periods (long term culture) by weekly feeding using long term culture medium (Iscove's Modified Dulbeccos Medium (IMDM - Gibco BRL Life Technologies Ltd), 10% heat-activated FCS, 10% heat-activated horse serum (HS), 1% hydrocortisone, 1% penicillin/streptomycin  $5 \times 10^{-7}\text{M}$  stock solution).

10

(i) First, following a certain time point, e.g. 24hr, from the initiation of CR3/43 treatment dilute cells in each well by adding 2mls of long term culture medium.

15

(ii) Feed wells weekly following removal of half of the growth medium.

(iii) Inspect wells using phase-contrast microscopy.

(F) Clonal Assays:

20 (i) After each time period following initiation of treatment, 300 $\mu\text{l}$  in culture medium of the non-adherent cells may be obtained as described above.

(ii) Add to the cell suspension in the culture medium above, 3 mls of methocult GFH4434 (StemCell Technologies, consisting of methylcellulose in IMDM, FCS, BSA, L-glutamine, rh stem cell factor, rh GM-CSF, rh IL-3 and rh erythropoietin).

25

(iii) Take 1.1 ml of cell mixture and plate in triplicate.

(iv) Incubate the plates at  $37^\circ\text{C}$  in a humidified petri dish with 5%  $\text{CO}_2$  and 5%  $\text{O}_2$  for 14 days.

(v) Inspect the wells before and after treatment with CR3/43 monoclonal antibody using phase-contrast microscopy.

30

(vi) After 14 days the cells (colonies) may be analysed by flow cytometry, confocal microscopy and/or PCR.



(vii) When carrying out the clonal assay alcohol aerosol should be avoided, as this gives rise to cell clumping or destruction, impeding the survival of both types of cells (treated and non-treated).

## 5 B. RESULTS

### **CD19 AND CD3 PANEL**

10 Treatment of blood samples in the device according to the present invention with monoclonal antibody to the homologous region of the  $\beta$ -chain of the HLA-DR antigen always decreased the relative number of CD19<sup>+</sup> cells. This marker is a pan B-cell antigen (see Tables). This antigen is present on all human B lymphocytes at all stages of maturation but is lost on terminally differentiated plasma cells. Hence, this is an indication that B cells were retrodifferentiating into undifferentiated cells.

15 The same treatment caused the relative number of CD3<sup>+</sup> cells to increase dramatically especially in blood of patients with B-CLL, which was always accompanied by an increase in the relative number in CD3<sup>+</sup>CD19<sup>+</sup> cells. CD3 is present on all mature T-lymphocytes and on 65%-85% of thymocytes. This marker is always found in association with  $\alpha$ - $\beta$ - or gamma/delta T-cell receptors (TCR) and together these complexes are important in transducing signals to the cell interior. Hence, this is an indication that B cells were retrodifferentiating into undifferentiated cells and then being committed to new differentiated cells, namely T cells.

25 A novel clone of cells appeared in treated blood of B-CLL patients co-expressing the CD19 and CD3 markers - i.e. CD19<sup>+</sup> and CD3<sup>+</sup> cells (see Chart 1, patient 2, 3 & 4 at 2hr, 6hr & 24hr of starting treatment), which treatment may be carried out in the device according to the present invention. Other patients with different conditions showed an increase in the relative number of these clones of cells. These cells were exceptionally large and heavily granulated and extremely high levels of CD19 were expressed on their cell membrane. The CD3 marker seems to be expressed on these cells at similar levels to those expressed on normal mature lymphocytes.

In Table 2, patient numbers 2, 3 and 4 are actually numbers representing the same patient and their delineation was merely to show the effect of treatment on blood with time (See Table 1 for experimental and clinical condition of this patient).

5

The CD19<sup>+</sup>CD3<sup>+</sup> clones in treated samples seem to decrease with time, reaching original levels to those determined in untreated sample at 2 hrs, 6 hrs and 24 hrs.

Another type of cell of the same size and granularity was detected in treated samples and these cells had high levels of CD19 expressed on their surface but were negative for the CD3 marker and rich in FC receptors. However, the relative number of these cells appeared to decrease in time. Of interest, at 24 hours treatment of blood sample (2, 3 and 4) there was a decrease in the relative number of CD19<sup>+</sup>CD3<sup>-</sup> cells in a group of cells that were initially observed to increase after 2 and 6 hrs treatment of blood samples. However, Coulter counts of WBC populations were reduced on treatment of blood with monoclonal antibody to the homologous region of the  $\beta$ -chain of the HLA-DR antigen. This finding suggests that this type of treatment gives rise to atypical cells that cannot be detected by Coulter (Table 1) but can be accounted for when measured by flow cytometry which counts cells on the basis of surface markers, size and granularity. Furthermore, these atypical cells were accounted for by analysing morphology using Wright's stain under a microscope. Flow cytometric charts of these phenomena are represented in Charts (1, 2, 3 & 4) and the immunophenotypic changes obtained on treatment of blood samples seems to suggest that CD19<sup>+</sup> and CD3<sup>+</sup> lymphocytes are an interconnected group of cells but remain distinct on the basis of CD19 and CD3 relative expression compared to stem cells.

In Table 2, patient numbers 5 and 6 represent the same patient but analysis of treated and untreated blood samples were monitored with time and at the same time (see Table 1).

Patients' blood with no B-cell malignancy showed similar trends of immunophenotypic changes when compared to blood of B-CLL patients but the changes were not to the same extent. However, the relative and absolute number of B-lymphocytes and MHC class II

positive cells in the blood of these patients are extremely low compared to those found in the blood of B-CLL patients.

Two brothers both with X-linked infantile hypogammaglobulinemia who were B cell  
5 deficient showed different immunophenotypic changes in the relative number of CD3<sup>+</sup>  
cells on treatment of their blood. The younger brother who was 2 months old and not ill,  
on treatment of his blood, showed a slight increase in the relative number of CD3<sup>+</sup> cells  
which was accompanied by a decrease in the relative number of CD3<sup>+</sup>CD19<sup>-</sup> cells. On  
the other hand, the other brother who was 2 years old and was extremely sick and with a  
10 relatively high number of activated T cells expressing the DR antigens showed a decrease  
in the number of CD3<sup>+</sup> cells on treatment of his blood. No other markers were used to  
measure other immunophenotypic changes that might have occurred because the blood  
samples obtained from these two patients were extremely small (Table 2, ID 43/BD and  
04/BD).

15 Patient 91 in Table 2 shows a decrease in the relative number of CD3<sup>+</sup> cells following  
treatment of blood which was accompanied by an increase in the relative number of CD3<sup>+</sup>  
CD19<sup>-</sup> cells. However, on analysis of other surface markers such as CD4 and CD8 (see  
Table 3) the patient was observed to have a high relative number of CD4<sup>+</sup>CD8<sup>+</sup> cells in  
20 his blood and this was noted prior to treatment of blood samples with monoclonal  
antibody to the  $\beta$ -chain of the DR antigen and these double positive cells decreased  
appreciably following treatment of blood. Furthermore, when further markers were  
analysed the relative number of CD3<sup>+</sup> cells were seen to have elevated (See Table 4).

25 An enriched preparation of B-lymphocytes obtained from healthy blood donors when  
treated with monoclonal antibody to the  $\beta$ -chain of DR antigens in a device according to  
the present invention showed a dramatic increase in the relative number of CD3<sup>+</sup> cells  
which were always accompanied by a decrease in the relative number of CD19<sup>+</sup> cells and  
by an increase in the relative number of CD19<sup>-</sup>CD3<sup>+</sup> cells. Further analysis using markers  
30 such as CD4 and CD8 show a concomitant increase in the relative number of these  
markers. However, an enriched preparation of T lymphocytes of the same blood donors  
when treated with the same monoclonal antibody did not show the same changes.

### CD4 AND CD8 PANEL

The CD4 antigen is the receptor for the human immunodeficiency virus. The CD4 molecule binds MHC class II antigen in the B2 domain, a region which is similar to the CD8 binding sites on class I antigens. Binding of CD4 to class II antigen enhances T cell responsiveness to antigens and so does the binding of CD8 to class I antigens. The CD8 antigens are present on the human suppresser/cytotoxic T-lymphocytes subset as well as on a subset of natural killer (NK) lymphocytes and a majority of normal thymocytes. The CD4 and CD8 antigens are coexpressed on thymocytes and these cells lose either markers as they mature into T-lymphocytes.

On analysis of the CD4 and CD8 markers - see below - and from a majority of blood samples presented in Table 2, a pattern of staining emerges which supports the presence of a retrodifferentiation process of B-lymphocytes into undifferentiated cells and the subsequent differentiation into T-lymphocytes.

CD4<sup>+</sup>CD8<sup>+</sup> cells, which are double positive cells, always appeared following treatment of blood samples with monoclonal antibody to the homologous region of the  $\beta$ -chain and these types of cells were markedly increased in the blood of treated samples of patients with B-CLL and which were absent altogether in untreated samples (See Table 3 and Charts 1, 2, 3 & 4). In the same specimens the relative number of single positive cells such as CD8<sup>+</sup> and CD4<sup>+</sup> cells was also noted to increase simultaneously. Furthermore, a decrease in the relative number of CD4<sup>+</sup>CD8<sup>-</sup> cells which, at least in the case of B-CLL correspond to B cells was noted to fall dramatically in treated samples when compared to untreated specimens which remained at the same level when measured with time. However, measurement of the relative number of CD4<sup>+</sup>CD8<sup>+</sup> cells with time in treated samples showed that there was a concomitant increase in the number of single positive cells with a decrease in the relative number of double positive cells. This type of immunophenotypic change is characteristic of thymic development of progenitor cells of the T-lymphocyte lineage in the thymus (Patient number 2,3 and 4). The CD4 antigen is present on the helper/inducer T- lymphocyte subsets (CD4<sup>+</sup>CD3<sup>+</sup>) and a majority of

normal thymocytes. However, this antigen is present in low density on the cell surface of monocytes and in the cytoplasm of monocytes and macrophages (CD3<sup>-</sup>CD4<sup>+</sup>).

The relative number of CD4<sup>+</sup> low cells was affected differently in different blood samples following treatment in the device according to the present invention. The relative number of this type of cells seems unaffected in blood samples of patients with B-CLL following treatment when compared to untreated samples. Such low levels of CD4 expression is found on monocytes and very early thymocytes.

- 10 Patient HIV<sup>+</sup>25 on treatment showed a substantial increase in the number of double positive cells expressing CD4 and CD8 simultaneously. On the other hand, patient 91 on treatment showed a decrease in this subtype of cells and the observation of such phenomenon is time dependent. The relative number of CD8<sup>+</sup> cells was observed to increase in untreated blood samples of patients with B-CLL when measured with time  
15 whereas the relative number of CD4<sup>+</sup> and CD4<sup>+</sup> low cells was observed to decrease at the same times (Table 3 patient 2, 3 and 4).

#### DR AND CD3 PANEL

- The DR markers are present on monocytes, dendritic cells, B-cells and activated  
20 T-lymphocytes.

- Treated and untreated samples analysed with this panel showed similar immunophenotypic changes to those obtained when blood samples were analysed with the CD19 and CD3 markers (see Table 2) and these antigens as mentioned earlier are pan  
25 B and T-cell markers respectively.

- Treatment of blood in a device according to the present invention with monoclonal antibodies seems to affect the relative number of DR<sup>+</sup> B-lymphocytes so that the level of DR<sup>+</sup> cells decrease. In contrast, the relative number of CD3<sup>+</sup> (T-cells) cells increase  
30 significantly (see Table 4 and Chart). Furthermore, the relative number of activated T cells increased in the majority of treated blood samples of patients with B-CLL and these types of cells were affected variably in treated samples of patients with other conditions.

Furthermore, the relative number of DR high positive cells appeared in significant numbers in treated samples of patients with B-CLL and a 6 day old baby with increased DR<sup>+</sup> CD34<sup>+</sup> blasts in his blood. However, it should be noted that the blasts which were present in this patient's blood were negative for T and B-cell markers before and after treatment but became more positive for myeloid lineage antigens following treatment. The relative number of CD3<sup>+</sup>DR<sup>+</sup> cells increased in the majority of treated blood samples and was proportional to increases in the relative number of CD3<sup>+</sup> cells (T-cells) and was inversely proportional to decreases in the relative number of DR<sup>+</sup> cells (B-cells).

#### 10 CD56&16 AND CD3 PANEL

The CD56&CD16 markers are found on a heterogeneous group of cells, a subset of lymphocytes known generally as large granular lymphocytes and natural killer (NK) lymphocytes. The CD16 antigen is expressed on virtually all resting NK lymphocytes and is weakly expressed on some CD3<sup>+</sup> T lymphocytes from certain individuals. This antigen is found on granulocytes in lower amount and is associated with lymphocytes containing large azurophilic granules. The CD16 antigen is the IgG FC receptor III.

A variable number of CD16<sup>+</sup> lymphocytes coexpress either the CD57 antigen or low-density CD8 antigen or both. In most individuals, there is virtually no overlap with other T-lymphocyte antigens such as the CD5, CD4, or CD3 antigens. The CD56 antigen is present on essentially all resting and activated CD16<sup>+</sup> NK lymphocytes and these subsets of cells carry out non-major histocompatibility complex restricted cytotoxicity.

Immunophenotyping of treated and untreated blood samples of B-CLL and some other patients with other conditions showed an increase in the relative number of cells coexpressing the CD56&CD16 antigens which were heavily granulated and of medium size (see Table 5 and Charts 1, 2, 3 & 4). These observations were also accompanied by a marked increase in the relative number of cells expressing the CD3 antigen only (without the expression of CD56 and CD16 markers) and cells coexpressing the CD56&CD16 and CD3 markers together.

In Table 5, patient numbers 2, 3, and 4 represent the same blood sample but being analysed at 2 hours, 6 hours and 24 hours respectively (before and after treatment). This sample shows that treatment of blood with monoclonal antibody to the homologous region of the  $\beta$ -chain of DR antigen seems to cause spontaneous production of CD56<sup>+</sup> and CD16<sup>+</sup> cells, CD3<sup>+</sup> cells and CD56<sup>+</sup> and CD16<sup>+</sup> CD3<sup>+</sup> cells and these observations were always accompanied by the disappearance of B-cell markers (CD19, DR, CD56, CD16<sup>+</sup>CD3<sup>+</sup>).

Onward analysis of this blood sample before and after treatment showed the levels of CD56<sup>+</sup> and CD16<sup>+</sup> cells to decrease with time and the level of CD3<sup>+</sup> cells to increase with time.

Blood samples of patient 7 with B-CLL, did not show any changes in the number of cells expressing the CD56, CD16 and CD3 antigens when compared to immunophenotypic changes observed in treated and untreated samples and this is because the amount of monoclonal antibody added was extremely low relative to the number of B lymphocytes. However, treatment of this patient's blood sample on a separate occasion with an appropriate amount of monoclonal antibody showed significant increases in the relative number of CD3<sup>+</sup>, CD56<sup>+</sup> & CD16<sup>+</sup> and CD56<sup>+</sup> and CD16<sup>+</sup> CD3<sup>+</sup> cells.

Blood samples of other patients with other conditions showed variable changes in the level of these cells and this seems to be dependent on the number of B-lymphocytes present in blood before treatment, duration of treatment and probably the clinical condition of patients.

#### **CD45 AND CD14 PANEL**

The CD45 antigen is present on all human leukocytes, including lymphocytes, monocytes, polymorphonuclear cells, eosinophils, and basophils in peripheral blood, thymus, spleen, and tonsil, and leukocyte progenitors in bone marrow.

The CD14 is present on 70% to 93% of normal peripheral blood monocytes, 77% to 90% of pleural or peritoneal fluid phagocytes. This antigen is weakly expressed on

granulocytes and does not exist on unstimulated lymphocytes, mitogen-activated T lymphocytes, erythrocytes, or platelets.

The CD45 antigen represents a family of protein tyrosine phosphatases and this molecule  
5 interacts with external stimuli (antigens) and effects signal transduction via the Scr-family  
members leading to the regulation of cell growth and differentiation.

Engagement of the  $\beta$ -chain of the DR antigens in treated blood samples especially those obtained from patients with B-CLL suggests that such a treatment affects the level of CD45 antigens on B-lymphocytes. The overall immunophenotypic changes that took place on stimulation of the  $\beta$ -chain of the DR antigen seem to give rise to different types of cells that can be segregated on the basis of the level of CD45 and CD14 expression as well as morphology as determined by forward scatter and side scatter (size and granularity respectively) and these results are presented in Table 6 and Charts (1, 2, 3 & 4). See also Figure 7 which demonstrates the appearance of CD45<sup>+</sup>CD14<sup>+</sup> cells after treatment with the CR3/43 antibody. These cells are not haemopoietic cells.

On treatment in the device according to the present invention the relative number of CD45 low cells (when compared to untreated samples) increased significantly and so did the relative number of cells co-expressing the CD45 and CD14 antigens. This type of immunophenotypic changes coincided with a decrease in the relative number of CD45 high cells (compared to untreated samples). However, this latter population of cells can be further divided on the basis of morphology and the degree of CD45 expression. One type was extremely large and had extremely high levels of CD45 antigen when compared to the rest of cells present in the charts (see charts 1, 2, 3 and 4). On analysis of this panel following treatment with time (see Table 6 patient 2, 3 and 4 and chart 1) the relative number of CD45<sup>+</sup> cells initially fell drastically with time to give rise to CD45 low cells. However, analysis of blood 24 hours later showed the opposite situation.

30 Samples 5 and 7 reveal opposite immunophenotypic changes to those obtained with other samples obtained from other B-CLL patients and this is because the samples were analysed at a much earlier incubation time with the monoclonal antibody. In fact the



sequential analysis of blood samples after treatment seems to suggest that the immunophenotypic changes undertaken by B lymphocytes is time dependent because it represents a stage of development and the immunophenotypic changes measured at time X is not going to be the same at time X plus (its not fixed once induced). However, these types of changes must be occurring in a more stringent manner in the body otherwise immunopathology would ensue. The effect of treatment of blood samples from other patients with no B-cell malignancy show variable changes in immunophenotypes of cells and this because B-lymphocytes are present in lower amount. However, treatment of enriched fractions of B-lymphocytes obtained from healthy blood donors show similar immunophenotypic changes to those obtained with B-CLL with high B lymphocyte counts.

#### CD8 AND CD3 PANEL

The CD8 antigenic determinant interacts with class I MHC molecules, resulting in increased adhesion between the CD8<sup>+</sup> T lymphocytes and the target cells. This type of interaction enhances the activation of resting lymphocytes. The CD8 antigen is coupled to a protein tyrosine kinase (p56lck) and in turn the CD8/p56lck complex may play a role in T-lymphocyte activation.

Treatment of blood samples in the device according to the present invention obtained from patients with B-CLL with monoclonal antibody to the B chain causes a significant increase in the relative number of CD3<sup>+</sup>CD8<sup>+</sup> and CD3<sup>+</sup> (highly likely to be CD4<sup>+</sup>CD3<sup>+</sup>) positive cells thus indicating more clearly that double positive cells generated initially are undergoing development into mature T-lymphocytes. This is a process that can be measured directly by CD19 and by DR and indirectly by CD8<sup>+</sup>CD3<sup>+</sup> antigens. Serial assessment of treated blood samples of the same patient with time seems to agree with a process which is identical to thymocyte development (Table 7, patient 2, 3 and 4 and Chart 1).

The relative number of CD8<sup>+</sup> cells increased with time in treated and untreated samples but to a higher extent in untreated samples. On the other hand, the relative number of CD8<sup>+</sup>CD3<sup>+</sup> cells decreased with time in untreated samples. However, the relative

number of CD3<sup>+</sup> cells increased in treated blood samples when measured with time and these types of cells highly correspond to CD4<sup>+</sup>CD3<sup>+</sup> single positive cells; a maturer form of thymocytes. In addition, since these samples were also immunophenotyped with other panels (mentioned above in Tables 3, 4, 5 and 6) the overall changes extremely  
 5 incriminate B cells in the generation of T lymphocyte progenitors and progenies.

Blood samples from a patient with B-CLL (number 2, 3 and 4 Tables 1, 2, 3, 4, 5, 6, 7) in separate aliquots were treated with nothing, PE conjugated monoclonal antibody to the homologous region of the  $\beta$ -chain of DR antigen and unconjugated form of the same  
 10 monoclonal antibody. On comparison of PE conjugated treatment clearly indicates no change in the relative number of CD3 positive cells and associated markers such as CD4 which have been observed in significant levels when the same blood sample was treated with unconjugated form of the antibody. However, an increase in the number of CD45  
 15 positive cells with no DR antigen being expressed on their surface was noted when measured with time (see Table 8). A finding that was similar to that noted in untreated samples when immunophenotyped with time (Table 6). Furthermore, the relative number of cells expressing CD45 low decreased in time, a phenomenon which was also noted in the untreated samples (when measured with time) of the same patient (see chart 1A).

## 20 **FAC analysis of cells derived from human buffy coat samples**

Treatment of buffy coat samples in a device according to the present invention with CR3/43 monoclonal antibody resulted in a greater incidence of CD34 (stem cell marker) and a lower incidence of CD19 (B-lymphocyte marker) - see Table 21.

25

Colony forming assays revealed that in the untreated samples, the predominant colony was of e

rythroid type, whereas the variation in colony type in the treated samples was much greater with the main colony type being pluripotent cell colonies, with  
 30 granulocytes/macrophages and megakaryocytes together with erythroid colony forming units also being observed.

These results demonstrate that the treated cells are much more capable of being able to differentiate along other lymphohaematopoietic pathways resulting in a variety of specialised cell lines.

5 C. COMPARISON OF THE EFFECT OF OTHER MONOCLONAL ANTIBODIES WITH DIFFERENT SPECIFICITY ON T-LYMPHOPHOIESIS

**CD19 AND CD3 PANEL**

10 Treatment of blood samples in a device according to the present invention with monoclonal antibody to the homologous region of the  $\alpha$ -chain of the DR antigen and the homologous region of MHC Class I antigens decreased the number of CD3<sup>+</sup> cells and increased the number of CD19<sup>+</sup> cells. Treatment of the same blood with monoclonal antibody to the homologous region of the  $\beta$ -chain of the DR antigen decreased the number of CD19<sup>+</sup> cells and increased the number of CD3<sup>+</sup> cells. Treatment with the  
15 latter monoclonal antibody with cyclophosphamide revealed the same effect (Table 14 patient 5/6 with B-CLL at 2hr treatment).

Onward analysis of CD19<sup>+</sup> and CD3<sup>+</sup> cells in the same samples revealed further increases in the relative number of CD3<sup>+</sup> cells only in blood treated with monoclonal antibody to  
20 the homologous region of the  $\beta$ -chain of DR antigen (Table 14 patient 5/6 at 24 hours following treatment). However, onward analysis (24 hours later patient 5/6 Table 14) of blood samples treated with cyclophosphamide plus monoclonal antibody to the  $\beta$ -chain of DR antigen show reversal in the relative number of CD19<sup>+</sup> and CD3<sup>+</sup> cells when compared to that observed at 2 hour incubation time under exactly the same condition.

25

In general, treatment of blood samples of the same patient with monoclonal antibody to the homologous region of the  $\alpha$  chain of the DR antigen or monoclonal antibody to the homologous of the  $\alpha$ -chain of the class I antigen shows an increase in the relative number of CD19<sup>+</sup> cells (pan B marker) when compared to untreated sample. The relative number  
30 of CD19<sup>+</sup>CD3<sup>-</sup> cells decreased slightly in blood samples treated with monoclonal antibody to the  $\alpha$ -chain of DR antigen or treated with monoclonal antibody to class I

antigens (see Table 14 & Charts 2, 3 & 4). Treatment of blood samples of patient 09 with monoclonal antibody to class I antigens increased the relative number of CD3<sup>+</sup> cells and decreased slightly the relative number of CD19<sup>+</sup> and CD19<sup>+</sup>CD3<sup>+</sup> cells. However, treatment of an enriched preparation of B-lymphocytes obtained from healthy blood donors with monoclonal antibody to the  $\beta$ -chain or  $\alpha$ -chain of DR antigen showed similar immunophenotypic changes to those obtained with patient with B-CLL.

Treatment of HIV<sup>+</sup> and IgA deficient patients with monoclonal antibody to the  $\beta$ -chain of the DR antigen increased the relative number of CD3<sup>+</sup> cells and decreased the relative number of CD19<sup>+</sup> cells. However, treatment of the same blood sample with monoclonal antibody to the homologous region of class I antigen did not produce the same effect. Treatment of blood samples obtained from patients (34/BD and 04/BD) with B-cell deficiency showed variable immunophenotypic changes when treated with monoclonal antibodies to the  $\beta$ -chain of the DR antigen, class I antigens and CD4 antigen.

#### CD4 AND CD8 PANEL

Blood samples analysed using the CD19 and CD3 panel (Table 14) were also immunophenotyped with the CD4 and CD8 panel (Table 15). Both panels seem to agree and confirm each other. Incubation for 2 hours of blood samples of patients with B-CLL (Table 15, patients 5/6 and 10, Charts 2, 3 & 4) with monoclonal antibody to the homologous region of the  $\beta$ -chain of the DR antigen or with this monoclonal antibody plus cyclophosphamide increased the relative number of CD8<sup>+</sup> and CD4<sup>+</sup> cells and cells coexpressing both markers. On the other hand, treatment of the same samples with monoclonal antibodies to the homologous region of the  $\alpha$ -chain of the DR antigen or the homologous region of the  $\alpha$ -chain of class I antigen did not produce the same effects.

Comparison of immunophenotypic trends obtained at 2 hours and 24 hours incubation periods with monoclonal antibody to the  $\beta$ -chain of the DR antigen plus cyclophosphamide revealed reverse changes in the relative number of CD4 and CD8 positive cells (Table 15, patient 5/6 with B-CLL at 2 hours and 24 hours) and such changes were in accordance with those obtained when the same blood sample was

analysed with the CD19 and CD3 panel (Table 14 the same patient). The later findings indicate that the subsequent differentiation is reversible as the undifferentiated cells can differentiate into T-lymphocytes or B-lymphocytes.

## 5 DR AND CD3 PANEL

The immunophenotypic changes obtained with DR and CD3 (Table 16) panel confirm the findings obtained with CD19 and CD3 panel and CD4 and CD8 panel (Tables 14 & 15 & Charts 2, 3 & 4) which followed treatment of the same blood samples with monoclonal antibodies to the homologous region of the beta- or alpha- side of the DR antigen or monoclonal antibody to class I antigens or monoclonal antibody to the  $\beta$ -chain of the DR antigen plus cyclophosphamide at 2 hour analysis.

From the results, it would appear that the monoclonal antibody to the homologous region of the  $\beta$ -chain of the DR antigen is extremely capable of driving the production of CD3 positive cells from DR<sup>+</sup> cells.

Furthermore, treatments such as those involving engagement of the  $\alpha$ -chain of DR antigens or engagement of the  $\beta$ -side of the molecule in conjunction with cyclophosphamide (prolonged incubation time) promoted increases in the relative number of CD19<sup>+</sup> cells or DR<sup>+</sup> cells.

## CD56&16 AND CD3 PANEL

Treatment of blood samples in a device according to the present invention, especially of those of patients with B-CLL with high B-lymphocyte counts with monoclonal antibody to the homologous region of the  $\beta$ -chain of the DR antigen increased the relative number of CD56&16 positive cells.

In these patients the relative number of CD3<sup>+</sup> and CD56<sup>+</sup> and CD16<sup>+</sup>CD3<sup>+</sup> cells also increased following treatment of blood samples with monoclonal antibody to the  $\beta$ -chain, confirming earlier observations noted with the same treatment when the same blood samples were analysed with CD3 and CD19 and DR and CD3 panels.

### CD45 AND CD14 PANEL

Blood samples treated in a device according to the present invention with monoclonal antibodies to the  $\beta$ - or alpha- chains of the DR antigen or to the  $\beta$ -chain plus cyclophosphamide or class I antigens were also analysed with the CD45 and CD14 panel (Table 18). The delineation of CD45 low, CD45 high and CD45 medium is arbitrary. Treatment of blood sample 5/6 (at 2 hours) with monoclonal antibodies to the  $\beta$ -chain of the DR antigen or with this monoclonal antibody plus cyclophosphamide generated CD45<sup>+</sup> low cells and increased the relative number of CD45<sup>+</sup> medium cells. However, the former treatment increased the relative number of CD45<sup>+</sup> high cells and the latter treatment decreased the relative number of CD45<sup>+</sup> medium cells and these changes appeared to be time dependent.

Blood samples of patient 5/6 and 10 (B-CLL) on treatment with monoclonal antibody to class I antigens showed a decrease in the relative number of CD45<sup>+</sup> medium cells and similar observations were noted in blood samples 09 and HIV<sup>+</sup> following the same treatment when compared to untreated samples. Treatment of blood samples of HIV<sup>+</sup> and IgA/D patients with monoclonal antibody to class I antigen increased the relative number of CD45<sup>+</sup> low cells when compared to untreated samples or samples treated with monoclonal antibody to the  $\beta$ -chain of the DR antigen. However, blood samples of these patients showed a decrease in the relative number of CD45<sup>+</sup> medium cells on treatment with monoclonal antibody to the homologous regions of the  $\beta$ -chain of the DR antigen. Medium CD45<sup>+</sup> cells increased in blood samples of IgA/D patient following monoclonal antibody to class I antigen treatment. Cells that were extremely large, heavily granular and expressing intense levels of CD45 antigen were noted in treated blood samples with monoclonal antibody to the homologous region of the  $\beta$ -chain of DR antigen of MHC class II antigens (see Charts 1, 2, 3 & 4).

### CD8 AND CD28 PANEL

The CD28 antigen is present on approximately 60% to 80% of peripheral blood T (CD3<sup>+</sup>) lymphocytes, 50% of CD8<sup>+</sup> T lymphocytes and 5% of immature CD3- thymocytes.

During thymocyte maturation, CD28 antigen expression increases from low density on most CD4<sup>+</sup>CD8<sup>+</sup> immature thymocytes to a higher density on virtually all mature CD3<sup>+</sup>, CD4<sup>+</sup> or CD8<sup>+</sup> thymocytes. Cell activation further augments CD28 antigen density. Expression of the CD28 also divides the CD8<sup>+</sup> lymphocytes into two functional groups.

5 CD8<sup>+</sup>CD28<sup>+</sup> lymphocytes mediate alloantigen-specific cytotoxicity, that is major histocompatibility complex (MHC) class I-restricted. Suppression of cell proliferation is mediated by the CD8<sup>+</sup>CD28<sup>-</sup> subset. The CD28 antigen is a cell adhesion molecule and functions as a ligand for the B7/BB-1 antigen which is present on activated B lymphocytes.

10 Treatment of blood samples in a device according to the present invention of patients (Table 19, patients 5/6 and 8) with B-CLL with monoclonal antibody to the homologous region of  $\beta$ -chain of the DR antigen increased the relative number of CD8<sup>+</sup>, CD28<sup>+</sup> and CD8<sup>+</sup>CD28<sup>+</sup> cells and all other types of treatments did not.

#### 15 CD34 AND CD2 PANEL

The CD34 antigen is present on immature haematopoietic precursor cells and all haematopoietic colony-forming cells in bone marrow, including unipotent (CFU-GM, BFU-E) and pluripotent progenitors (CFU-GEMM, CFU-Mix and CFU-blast). The

20 CD34 is also expressed on stromal cell precursors. Terminal deoxynucleotidyl transferase (TdT)<sup>+</sup> B- and T-lymphoid precursors in normal bone are CD34<sup>+</sup>. The CD34 antigen is present on early myeloid cells that express the CD33 antigen but lack the CD14 and CD15 antigens and on early erythroid cells that express the CD71 antigen and dimly express the CD45 antigen. The CD34 antigen is also found on capillary endothelial cells

25 and approximately 1% of human thymocytes. Normal peripheral blood lymphocytes, monocytes, granulocytes and platelets do not express the CD34 antigen. CD34 antigen density is highest on early haematopoietic progenitor cells and decreases as the cells mature. The antigen is absent on fully differentiated haematopoietic cells.

30 Uncommitted CD34<sup>+</sup> progenitor cells are CD38<sup>-</sup>, DR<sup>-</sup> and lack lineage-specific antigens, such as CD71, CD33, CD10, and CD5, while CD34<sup>+</sup> cells that are lineage-committed express the CD38 antigen in high density.

Most CD34<sup>+</sup> cells reciprocally express either the CD45RO or CD45RA antigens. Approximately 60% of acute B-lymphoid leukaemia's and acute myeloid leukaemia express the CD34 antigen. The antigen is not expressed on chronic lymphoid leukaemia (B or T lineage) or lymphomas. The CD2 antigen is present on T lymphocytes and a subset of natural killer lymphocytes (NK).

The results are shown in Charts 2, 3 and 4.

10 Analysis of blood samples of a patient with B-CLL (Table 20, patient 5/6 at 2hours) after treatment with monoclonal antibodies to the  $\beta$ -chain of the DR antigen or the  $\alpha$ -chain of the same antigen revealed marked increases in the relative number of CD34<sup>+</sup> and CD34<sup>+</sup>CD2<sup>+</sup> cells after treatment with the former antibody. Since the same blood samples were immunophenotyped with the above mentioned panels (see Tables 14 to 19 ),  
15 for other markers the increase in the relative number of CD34<sup>+</sup> and CD34<sup>+</sup>CD2<sup>+</sup> cells observed here seems to coincide with increases in the relative number of CD4<sup>+</sup>CD8<sup>+</sup>, CD8<sup>+</sup>CD3<sup>+</sup> and CD4<sup>+</sup>CD3<sup>+</sup> single positive (SP) cells. Furthermore, these findings which seem exclusive to engagement of the  $\beta$ -chain of the HLA-DR antigen, are in direct support that the process is giving rise to T-lymphopoiesis via B lymphocyte regression.

20 On analysing the same treatment 24 hours later the CD34<sup>+</sup> cells seemed to decrease in levels to give rise to further increase in the relative number of T lymphocytes. The process of retrodifferentiation that initially gave rise to T-lymphopoiesis can be reversed to give rise to B-lymphopoiesis. The former phenomenon was observed at 2 hours  
25 incubation time with monoclonal antibody to the  $\beta$ -chain of the HLA-DR antigen plus cylophosphamide, whereas the latter process was noted at 24 hours incubation time with the same treatment in the same sample (Chart 2).

Treatment of blood samples of HIV<sup>+</sup> patient (Table 20 patient HIV+) with monoclonal  
30 antibody to the  $\beta$ -chain of the HLA-DR antigen markedly increased the relative number of CD34<sup>+</sup> and CD2<sup>+</sup>CD34<sup>+</sup> cells and so did treatment of the same blood sample with



monoclonal antibody to the  $\beta$ -chain of the HLA-DR antigen and monoclonal antibody to the  $\alpha$ -chain of the same antigen when added together. However, treatment of this blood sample with monoclonal antibody to the  $\alpha$ -chain of the HLA-DR antigen did not affect the level of  $CD34^+$  cells. Treatment of blood samples obtained from a 6-day old baby (BB/ST Table 20) who was investigated at that time for leukaemia and who had very high number of atypical cells (blasts) in his blood with monoclonal antibody to the  $\beta$ -chain of the HLA-DR antigen, or monoclonal antibody to the  $\alpha$ -chain of the same antigen or both monoclonal antibodies added together resulted in the following immunophenotypic changes.

On analysis of untreated blood samples the relative number of  $CD34^+$  and  $DR^+$  cells were markedly increased and on treatment with monoclonal antibody to the  $\beta$ -chain the relative number of  $CD34^+$  cells further increased but were noted to decrease on treatment with monoclonal antibody to the  $\alpha$ -chain of the HLA-DR antigen or treatment with monoclonal antibodies to the  $\alpha$  and  $\beta$ -chains of the molecule when added together. However, the latter treatment increased the relative number of  $CD34^+CD2^+$  cells and the opposite occurred when the same blood sample was treated with monoclonal antibody to the  $\beta$ -chain of the HLA-DR antigen alone. On analysis of treated and untreated blood aliquots of the same patient 24 hours later the relative number of  $CD34^+$  decreased with all above mentioned treatments except it was maintained at a much higher level with monoclonal antibody to the  $\beta$ -chain of the HLA-DR antigen treatment. The latter treatment continued to decrease the relative number of  $CD34^+CD2^+$  cells 24 hours later.

These results indicate that engagement of the HLA-DR antigen via the  $\beta$ -chain promotes the production of more  $CD34^+$  cells from  $CD2^+CD34^+$  pool or from more mature types of cells such as B-lymphocytes of patients with B-CLL and these results indicate that this type of treatment promotes retrodifferentiation. However, immunophenotyping of blood samples 24 hours later suggests that these types of cells seem to exist in another lineage altogether and in this case cells seem to exist or rather commit themselves to the myeloid lineage which was observed on analysis of treated blood sample with the CD7 and CD13&33 panel.

Morphology changes immunophenotypic characteristics of B-lymphocytes of B-CLL and enriched fractions of healthy individuals (using CD19 beads) on treatment with monoclonal antibodies to homologous regions of the  $\beta$ -chain of MHC class II antigens.

5 These were accompanied by a change in the morphology of B-lymphocytes. B-lymphocytes were observed colonising glass slides in untreated blood smears were substituted by granulocytes, monocytes, large numbers of primitive looking cells and nucleated red blood cells. No mitotic figures or significant cell death were observed in treated or untreated blood smears.

10

The results of Table 20 also demonstrate a further important finding in that according to the method of the present invention it is possible to prepare an undifferentiated cell by the retrodifferentiation of a more mature undifferentiated cell.

15 D. MICROSCOPE PICTURES

In addition to the antigen testing as mentioned above, the method of the present invention was followed visually using a microscope. The device of the present invention may be programmed to track the changes automatically by tracking means.

20

In this regard, Figure 8 is a microscope picture of differentiated B cells before the method of the present invention. Figure 9 is a microscope picture of undifferentiated cells formed by the retrodifferentiation of the B cells in accordance with the present invention wherein the agent was a monoclonal antibody to the homologous regions of the  $\beta$ -chain of HLA-  
25 DR antigen. The undifferentiated cells are the dark stained clumps of cells. Figure 10 is a microscope picture of the same undifferentiated cells but at a lower magnification.

Figures 8 to 10 therefore visually demonstrate the retrodifferentiation of B cells to undifferentiated stem cells by the method of the present invention.

30

Figure 11 is a microscope picture of differentiated B cells before the method of the present invention. Figure 12 is a microscope picture of undifferentiated cells formed by

the retrodifferentiation of the B cells in accordance with the present invention wherein the agent used was a monoclonal antibody to the homologous regions of the  $\beta$ -chain of HLA-DR antigen. Again, the undifferentiated cells are the dark stained clumps of cells. Figure 13 is a microscope picture of the formation of differentiated granulocyte cells from the same undifferentiated cells of Figure 12.

Figures 11 to 13 therefore visually demonstrate the retrodifferentiation of B cells to undifferentiated stem cells by the method of the present invention followed by commitment of the undifferentiated cells to new differentiated cells being of a different lineage as the original differentiated cells.

These microscopy experiments have also been performed with blood from BCLL patients, treated with the CR3/43 monoclonal antibody as described above. As discussed above, blood from BCLL cells is a useful aid in studying the retrodifferentiation process because the blood contains higher than normal numbers of B lymphocytes. The results are shown in detail in Figures 14 to 17.

Figure 14 shows at two different magnifications, an untreated blood sample from a BCLL patient. The untreated B lymphocytes (blue cells) show typical morphology, i.e. condensed chromatin structure and sparse cytoplasm. The remaining cells are erythrocytes (red blood cells).

Treatment of blood samples with antibody CR3/43 leads initially to clustering of B lymphocytes into aggregates (Figure 15).

The clustered B cells gradually lose their typical morphology, characterized by the formation of cobblestone-like- cell areas, decondensation of chromatin structure, appearance of prominent nucleoli, enlargement of cell volume and cytoplasmic basophilia typical of undifferentiated cells (Figure 16). Relaxed (decondensed) chromatin structure is an important feature of undifferentiated cells as compared to differentiated cells. This is likely to be due to a need for more extensive access to transcriptional units to determine changes in gene expression required for commitment

along a given cell lineage. By contrast, it is well known that more differentiated cells have a more condensed chromatin structure since only a small amount of chromatin needs to be transcriptionally active.

- 5 The appearance of undifferentiated cells is always accompanied by the appearance of cells (17A to 17J) with differentiated morphology. Importantly, these cells could not have arisen by proliferation, since (i) the incubation time was too short for one or more complete cell divisions to take place (ii) no mitotic figures are seen and (iii) the absolute number of leucocytes remained the same before and after treatment. Furthermore less  
10 differentiated progenitors were seen in association with their more differentiated progenies (see the myeloid precursor in Figure 17J), indicating that these specialised cells arose by differentiation.

Micrographs Figure 17A to 17J show the types of differentiated cells seen following  
15 treatment of B-CLL lymphocytes with CR3/43 monoclonal antibodies: Platelets (Pl) – Figure 17A, Neutrophils (Ne) – Figure 17B, Eosinophils (Eso) – Figure 17C, Megakaryocytes (Meg) – Figure 17D, Basophils (Ba) – Figure 17G, Lymphocytes (Ly) – Figure 17H, Monocytes (Mo)- Figure 17I and Myeloid progenitors (Mp) – Figure 17J. Also seen were erythroid progenitors and macrophages (data not shown).

20 Thus, in summary, these microscopy results show changes in B cell morphology in samples from BCLL patients, who have high levels of mature B lymphocytes. The microscopy pictures show changes in the morphology of the B lymphocytes, which initially cluster, followed by the appearance of various cells with a graded range of  
25 morphologies from progenitor cells to differentiated cells (neutrophils, basophils, eosinophils, megakaryocytes, platelets, lymphocytes, macrophages, granulocytes, stab granulocytes and stromal-like cells).

In addition, and very importantly, the presence of erythroid and myeloid progenitors is  
30 seen (Figure 17J – and data not shown). The myeloid progenitor is clearly distinguishable morphologically from the other cells, being larger and with a distinct nuclear morphology as well as containing cytoplasmic granules.

The microscopy data therefore support morphologically what the flow cytometry data indicate in terms of cell surface markers. These data allow one to conclude that treatment of B lymphocytes with an antibody to MHC HLA-DR  $\beta$  chain results in a decrease in the numbers of B lymphocytes and an increase in the number of cells of other haemopoietic lineages including immature precursor cells.

The retrodifferentiation of T cells treated with an antibody to an MHC class II  $\alpha$ -chain (monoclonal antibody TAL.1B5) to undifferentiated stem cells by the method of the present invention followed by commitment of the undifferentiated cells to new differentiated cells being of a different lineage as the original differentiated cells was also followed by microscopy (data not shown).

#### E. ANALYSIS OF VDJ RECOMBINATION REARRANGEMENTS IN RETRODIFFERENTIATED LYMPHOCYTES

By way of background, the differentiated cells used in these experiments (B lymphocytes or cells with certain properties of T lymphocytes) have genes which have already undergone rearrangement to encode a mature Ig or a TCR, respectively. In the process of rearranging, intermediate portions of DNA that are not part of the final, expressed TCR or Ig gene, primarily DNA which is between the variable (V) region encoding segment and the constant (C) region-encoding segment of these receptors, are spliced out of the genome. These excised fragments are retained in the cell in the form of extrachromosomal DNA. For the cells to truly retrodifferentiate, the excised DNA would be reinserted into the genome, placing the cells in a state similar to that preceding their original differentiation. Because of this, a probe complementary to a sequence in the rearranged gene will be expected to hybridize to a larger DNA restriction fragment when the DNA has returned to its unrearranged or germ line state as compared to the rearranged DNA that characterizes the differentiated state.

30

##### 1. Rearrangement of TCR genes in Daudi cells

In the experiment resulting in the Southern blot shown in Figure 18, a well-known cell line, Daudi, a B-cell lymphoma with one rearranged TCR gene (and the other deleted), was used. Genomic DNA was prepared from Daudi cells and digested with EcoRI, subjected to gel electrophoresis and probed with a labeled TCR  $\beta$ -chain DNA probe.

- 5 Daudi cells were used rather than B lymphocytes purified from human patients because these cells are clonally related and form a homogenous cell population with the same gene rearrangements that can be clearly viewed by Southern blotting of digested genomic DNA. In a normal blood sample, different cells have different rearrangements and so a Southern blot would appear as a smear.

10

A functional gene encoding the TCR  $\beta$ -chain is assembled in lymphocytes by a series of somatic rearrangements that occur during lymphocyte maturation to bring together a V segment, a D segment and a J segment. A very clear explanation of these rearrangement processes is given in Genes VI, Lewin, Oxford University Press, 1997  
 15 (pages 1994-1023) – a standard undergraduate textbook. Particular pages are cited below.

- Firstly, a D segment is joined by a recombination process to one of several J segments in a D-J joining reaction. Then, one of the many possible V segments(<60) is joined to  
 20 the resulting DJ segment (V-D joining) to form a complete TCR  $\beta$ -chain gene. The constant region gene is immediately downstream of the rearranged VDJ segment, although there may be intervening J segments which are spliced out during RNA processing to bring the constant gene exon into proximity with the rearranged VDJ gene segment (Lewin, p998).

25

- In human cells, there are two different TCR  $\beta$ -chain constant region gene segments, denoted C $\beta$ 1 and C $\beta$ 2, present at two different loci, each of which is preceded by a cluster of six or seven joining region (J $\beta$ ) gene segments (J $\beta$ 1 and J $\beta$ 2) and one D segment (D $\beta$ 1 and D $\beta$ 2) (see Figure 1, Toyonaga *et al.*, 1985, Proc. Natl. Acad. Sci.  
 30 USA 82: 8624-8628 and Lewin, p1017).

- The recombination events which lead to the V, D and J-C segments being brought into proximity are catalysed by a multitude of proteins, including RAG-1 and RAG-2 which recognise nonamer and heptamer sequences present at the recombining ends of the V, D and J-C gene sequences. Depending on the orientation of these nonamer/heptamer sequences, recombination results either in an inversion or a deletion. Both types of events will result in a change in the restriction enzyme fragment pattern of the genomic DNA. Furthermore, a deletion event does not necessarily result in complete loss of the excised fragment. Rather, the ends of the excised fragment are rejoined to produce a circle of DNA which remains in the cell (Okazaki *et al.*, 1987, Cell 49: 477-85; Davis *et al.*, 1991, J. Exp. Med. 173: 743-6; Livak and Schatz, 1996, Mol. Cell Biol. 16: 609-18; Harriman *et al.* 1993, Annu Rev Immunol. 11:361-84). Each gene segment, of course, has two alleles since cells have a diploid chromosome complement.
- In the normal germline state, the C $\beta$ 1 and C $\beta$ 2 genes are arranged as shown in Figure 1, Toyonaga *et al.*, 1985. A restriction digest of genomic DNA with EcoRI will generate two relevant bands detectable by the probe used in the experiment (the probe is a labelled DNA fragment derived from C $\beta$ 1 which also hybridises to C $\beta$ 2 due to a high degree of sequence homology): (i) a 12 kb band containing C $\beta$ 1 sequence; and (ii) a 4 kb band containing C $\beta$ 2 sequence. This germline configuration is seen in undifferentiated immature cells (lane A of Figure 18) This germline configuration is also perfectly illustrated by lane 3 (2 hours with CR3/43 antibody) of Figure 18, giving an identical pattern to that of lane A.
- In the differentiated state, both alleles of C $\beta$ 1 and C $\beta$ 2 genes are rearranged such that there is no longer a 12 kb fragment at the C $\beta$ 1 locus or a 4 kb fragment at the C $\beta$ 2 locus. In fact, no hybridising fragment derived from the C $\beta$ 1 locus is present on the gel (this is due to deletion of the hybridising sequence from both C $\beta$ 1 alleles as a result of recombination). As for the C $\beta$ 2 locus, there are actually now two major bands corresponding to different "alleles" resulting from rearrangements on both chromosomes. The largest band, which is smaller than 4 kb, corresponds to a fragment

of one of the two rearranged alleles. The lowest band is a fragment of the other rearranged allele. The intermediate minor band is probably derived from a subclone of Daudi cells with a different rearrangement – hence its presence in a submolar amount to either allele. Nonetheless, the rearranged state is very clearly shown in lane 1 where  
 5 both major bands are clearly visible.

2 hours with the negative control antibody (TAL.1B5) which binds to the  $\alpha$ -chain of MHC-DR actually results in the loss of the upper band, whereas the lowest band has a similar intensity to the untreated cells in lane 1 (see lane 2). A possible explanation for  
 10 this is that the cells are differentiating, further resulting in a further recombination event at the C $\beta$ 2 locus of one allele, which leads to loss of C $\beta$ 2 sequences. This is entirely consistent with known phenomena.

24 hours with the negative control antibody appears to restore the three bands seen in  
 15 the untreated cells (see lane 4). However the bands actually migrate at a lower position than the bands seen in lane 2. It is not quite clear how this has arisen. A possible explanation is that reintegration of deleted sequences has occurred, consistent with the looping-out-excision-reintegration model (Malissen *et al.*, 1986, Nature 319: 28-32). Nonetheless, neither result seen with the TAL.1B5 antibody at 2 hours or 24  
 20 hours is indicative of a rearrangement to the germline pattern. Lanes 2 and 4 actually represent a negative control – the antibody to the  $\alpha$ -chain does not result in restoration of the germline sequences.

By contrast, the results obtained with a monoclonal antibody (CR3/43) to the  $\beta$ -chain  
 25 of MHC-DR after two hours show a pattern of bands that correspond to the germline configuration, namely a 12 kb band and a 4 kb band (compare lane 3 with lane A). In other words, these results show that the germline restriction pattern at the C $\beta$ 1 and C $\beta$ 2 loci has been restored for all alleles.



From these results we conclude that the pattern of bands seen in lane 3 are indicative of a rearrangement of the genomic DNA of the differentiated cells to regenerate the germline configuration.

5 The importance of this finding should not be understated. A genomic rearrangement, including deletions, can be reversed to restore the genome to the state in which it existed before the differentiation process took place. The most likely explanation is that the inversion caused by the rearrangement of the C $\beta$ 2 alleles during differentiation has been reversed, and the deletion of the C $\beta$ 1 sequence that caused loss of the 12 kb  
10 bands has also been reversed. The source of the missing C $\beta$ 1 sequence is likely to be episomal circular DNA present in the nucleus from the original deletion event. The existence of this circular DNA has been catalogued in the prior art (see references cited above). Nonetheless, the precise mechanism by which this restoration of the germline genome has occurred is not important. What *is* important is that it has  
15 occurred.

A continued incubation with the monoclonal antibody (CR3/43) to the  $\beta$ -chain of MHC-DR for 24 hours in the device according to the present invention results in a more complex banding pattern (lane 5). However these bands do not represent the  
20 same bands as in the untreated control. In particular, fragments of about 12 kb that hybridise to the probe are still present ("C $\beta$ 2 alleles"). Further, it is important to appreciate that the bands marked "C $\beta$ 2 alleles" do not correspond to the smaller than 4 kb band seen in the untreated control (lane 1). The most likely explanation for the results seen in lane 5 is that a secondary rearrangement process has occurred since the  
25 hybridisation pattern resembles that of T-cells in that it is characterised by a rearranged TCR gene (this explanation is consistent with the flow cytometry data showing an increase in cells having cell markers characteristic of T cells). Nonetheless, regardless of the precise molecular explanation, the results seen in lane 5 at 24 hours exposure to the CR3/43 antibody are supportive of the results obtained at 2 hours exposure in lane  
30 3.

## 2. Rearrangement of Ig gene in B-CLL cells

The Southern blot shown in Figure 19B was obtained using peripheral blood cells from patients with chronic lymphocytic leukemia (B-CLL). Genomic DNA was prepared from these largely monoclonal B cells and digested with BamHI and HindIII, subjected to gel electrophoresis and probed with a labeled TCR DNA probe. These B-CLL cells were treated for 24 hours with the CR3/43 (anti class II MHC chain of HLA-DR, DP and DQ) which was described above. The blots were probed with a radiolabeled Ig J region probe. The two bands obtained from the untreated cells in lane A, represent the two rearranged Ig alleles (paternal and maternal). These bands did not appear in lane B which shows the pattern 24 hours after antibody treatment of cells. In their place appeared a 5.4 kb band characteristic of the germ line Ig gene.

In another experiment, shown in Figure 19A, cells were left untreated or treated for the times indicated with the anti-class II MHC  $\beta$ -chain antibody. The Ig VDJ region was amplified by PCR in the differentiated (control) and antibody-treated B-CLL cells (left half of gel). This generated a VDJ amplification product from the untreated cells. However, no such band was observed in the antibody-treated cells because, as a result of insertion of the excised genomic DNA, this "germ line" DNA configuration was not susceptible to PCR amplification using the particular primers for VDJ. A similar experiment (right side of gel) allowed me to visualize the behavior of a control, housekeeping, gene encoding  $\beta$ -actin. There was no difference in the  $\beta$ -actin PCR amplification product, regardless of treatment. Thus, this "control" gene did not appear to be affected by the retrodifferentiation process that caused profound alterations in the Ig gene of the same cells under the same conditions.

The results presented above show that treatment of cells with an agent that engages an appropriate cell surface receptor induces retrodifferentiation of these cells that is proven at the molecular level (and monitored) by observing the retrogression of the rearrangements of chromosomal DNA that characterize the differentiated state. Thus, it is concluded on the basis of the molecular genetic and morphological evidence that cells of the B lymphocyte lineage, treated with an agent (mAb) that engages the class

II MHC  $\beta$ -chain, undergo retrodifferentiation. By contrast, the same cells treated with antibodies that engage class II  $\alpha$ -chain are not similarly induced to retrodifferentiate. If anything, they appear to differentiate (forward) along the B cell pathway.

## 5 F. FURTHER STUDIES ON RETRODIFFERENTIATION OF B LYMPHOCYTES

10 FACsVantage purified BCLL cells (95% pure B cells) from BCLL patients were treated with the CR3/43 antibody in a device according to the present invention as described above and the cells processed by flow cytometry. The results shown below in Table A confirm further the results obtained above. A significant increase in the number of CD34<sup>+</sup> cells was obtained together with a large reduction in the number of cells having cell surface markers characteristic of the B lymphocyte lineage (CD19, CD20 and CD22). An important point to note from Table A is that it also shows an  
15 increase in the number of cells that are both CD34 negative and lineage negative. These undifferentiated cells are not committed to the haematopoietic lineage and precede CD34<sup>+</sup> stem cells in differentiation. Further, examination of samples by light microscopy showed a range of adherent cell types having morphological characteristics of non-haematopoietic cells.

20

Table A

Marker	0 hr	2 hr	24 hr
CD20	73	67	16
CD14	0	3	23
CD34	0	1	23
CD7	0	2	0
CD16	8	3	2
CD19	95	71	1
CD22	5	3	2
CD33	0	0	0
CD3	0	0	0

The loss of CD19 cell surface markers accompanied by the appearing of CD34 cell surface markers on the same cell has also been demonstrated and recorded on video in real time using confocal microscopy. B-lymphocytes before the addition of CR3/43 mab stained green with a FITC conjugated monoclonal antibody to CD19. After the addition of CR3/43 mab, cells lost their green fluorescence and began to stain red with a PE/Cy5 (or quantum red) conjugated monoclonal antibody to CD34 but not green (see Figure 23 which shows two still images from the timelapse video). The results clearly confirm that during B lymphocyte retrodifferentiation, lineage specific markers such as CD19 are lost whilst a stem cell marker such as CD34 is re-expressed.

#### G. OTHER AGENTS THAT INDUCE RETRODIFFERENTIATION OF B LYMPHOCYTES TO HAEMATOPOIETIC STEM CELLS

Initial studies actually identified three agents – granulocyte/monocyte-colony stimulating factor (GM-CSF), erythropoietin and mAb CR3/43. A preparation of enriched, purified, normal B lymphocytes was treated in a device according to the present invention with one of these three agents in a similar manner to that described for CR3/43 and TAL.1B5 above and treated samples examined by flow cytometry as described above. Compared with the negative control, all three samples treated with either GM-CSF, erythropoietin or mAb CR3/43 showed changes consistent with retrodifferentiation. In particular, all three agents increased the relative number of CD34<sup>+</sup> cells in the cell population (see Figure 20). The greatest effect, however, was seen with CR3/43 and consequently, this agent was selected for use in the more detailed studies presented herein.

#### H. PROPERTIES OF HAEMATOPOIETIC STEM CELLS PRODUCED BY THE RETRODIFFERENTIATION PROCESS

### **Colony forming assays**

To confirm that the CD34<sup>+</sup> cells observed by flow cytometry and the undifferentiated cells identified by microscopy had the properties of undifferentiated haematopoietic cells, blood samples treated with an antibody to the class II MHC  $\beta$ -chain (CR3/43 – see above) were subjected to colony forming assays – a standard method known in the art for assessing the capabilities of primitive haematopoietic cells. The colony forming assays may be conducted automatically in the device according to the present invention as part of the tracking mechanism.

*In vitro* clonal assays for hematopoietic stem cell allows the quantification of primitive progenitor cells that possess the ability to proliferate, differentiate and develop into phenotypically and functionally mature myeloid and/or erythroid cells. For example in the presence of growth factors stem cell when seeded/immobilised in soft-gel matrix *in vitro* are capable of clonal growth (proliferation) and differentiation.

Figure 21 is a colony assay of stem cells produced according to the methods of the invention, using inverted bright-field microscopy. In this assay B cells obtained from buffy coat of healthy blood donors were treated with CR3/43 mab and then subjected to colony assays as described in the materials and methods section.

Panels (a) to (e) in Figure 21 show:

- a) Bright field microscopy of culture dish viewed at x 3 magnification showing erythroid, myeloid and mixed (consisting of mature myeloid and erythroid cells) colonies which can be seen readily even by the naked eye. Each colony arose from a single haematopoietic stem cell by proliferation and subsequent differentiation.
- b) MIX-CFC this colony arose from a single multi-potent haematopoietic stem cell (stem cells capable of giving rise to cells of myeloid and erythroid lineages).
- c) M-CFC this colony consists of macrophages.
- d) GM-CFC this colony consist of the myeloid lineage including macrophages, granulocyte and megakaryocytes.
- e) BFU-E this colony consists of cells belonging to the erythroid lineage such as normoblasts and non-nucleated red cells. The red colouration of cells shows that

they are well hemogloblinized. The large size of this colony indicates that it arose from an extremely primitive stem cell.

The same results were obtained with B-CLL cells (data not shown). Untreated B cells did not give rise to haematopoietic colonies (data not shown). These results therefore demonstrate the presence of viable haematopoietic stem cells in blood samples treated with monoclonal antibody CR3/43 to the class II MHC  $\beta$ -chain but not in untreated blood samples.

## 10 Long Term culture

The long-term assay examines the self-renewal potential of haematopoietic stem cells. In this culture most components of bone marrow haematopoiesis are reproduced *in vitro*. The important feature of this culture is sustained haematopoiesis, which occurs in the absence of added growth factors. In this assay the process of haematopoiesis is absolutely dependent upon the establishment of an adherent layer of bone marrow derived stromal cells. Stromal cells (consisting of a variety of non-haematopoietic cells e.g., fibroblast, fat cells and including all cell types belonging to the mesenchymal system) support haematopoiesis by providing the appropriate environment (secretion of growth factors and synthesis of extracellular matrix) to promote the survival, self-renewal, proliferation and differentiation of the stem cells.

In this assay, treatment of B cells obtained from buffy coats of healthy blood donors (the same results were obtained with B-CLL cells) with CR3/43 mab gave rise to the formation of an adherent cell layer within hours of adding the antibody which, also increased with time.

The adherent layer consisted of stromal cells (blanket cells, consisting mainly of fibroblast/mesenchymal-type cells/light refringent large cells when viewed with inverted bright field microscopy – see Figure 22) which supported the growth and development of haematopoietic cells up to 12 weeks and longer (these cells show

intimate contact with haematopoietic cells). Also visible in the adherent layer are groups of primitive haematopoietic cells (also known as cobblestone areas/clusters of dark appearing cells) which are the origin of prolonged production of haematopoietic cells.

5

The non-adherent layers which are on top of the stromal layer (clusters of bright appearing cells) consisting of small round cells forming clusters of haematopoietic foci. This layer contains stem cells and also more committed progenitors of the haematopoietic system. The non adherent layer was capable of giving rise to MIX-CFC, GM-CFC, M-CFC, BFU-E (as determined using the clonal assay) and CFU-F (colony forming unit-fibroblast) (when sub-cultured with long term culture medium).

10

#### I. RT-PCR OF CELLS TREATED WITH AN ANTIBODY TO THE $\beta$ -CHAIN OF HLA-DR.

15

Gene transcription was measured in Ramos (B lymphoma) and K562 (erythroid leukaemia) cells treated with the CR3/43 mab for the CD34, c-kit (ligand of stem cell factor),  $\epsilon$ -haemoglobin (embryonic form of haemoglobin) and  $\beta$ -actin genes.

#### 20 Methods

25

mRNA was extracted before and after treatment with CR3/43 mab using RNeasy (Qiagen). mRNA were subjected to hexamer priming reverse transcription by incubating at room temperature for 5 mins with 4  $\mu$ l standard buffer, 2  $\mu$ l dNTPs, 1  $\mu$ l RNASIN, 1  $\mu$ l reverse primer (random hexamer primer) and 1  $\mu$ l MMLV reverse transcriptase enzyme. This mixture was further incubated for 1 hr at 38°C. Mixtures were then subjected to PCR under standard conditions using primers designed to amplify CD34, c-kit,  $\epsilon$ -haemoglobin and  $\beta$ -actin sequences. Primers were synthesised at the Randall Institute Kings College according to published data.

## Results

The results obtained show that whereas the levels of  $\beta$ -actin mRNA did not change, the levels of CD34, c-kit and  $\epsilon$ -haemoglobin mRNA all increased significantly following treatment with the CR3/43 mAb. The results for CD34 and c-kit provide further support for the data detailed above that demonstrate the retrodifferentiation of B lymphocytes to produce haemopoetic stem cells.

The results obtained for the  $\epsilon$ -haemoglobin are even more interesting since  $\epsilon$ -haemoglobin is normally only expressed in embryonic cells. It is therefore possible that treatment with the CR3/43 mAb not only gives rise to haematopoietic stem cells but also to even more primitive undifferentiated cells such as embryonic stem cells.

### 15 J. SUMMARY

In short, the device according to the present invention may be used in the retrodifferentiation of differentiated cells into stem cells. The examples describe *in vitro* experiments that reveal extremely interesting, seminal findings regarding the ontogeny and development of T and B lymphocytes which can be utilised in the generation of stem cells to affect lymphohaematopoiesis in peripheral blood samples in a matter of hours.

Treatment of peripheral blood samples obtained from patients with B-cell chronic lymphocytic leukaemia's (B-CLL) with high B lymphocyte counts, with monoclonal antibody to the homologous region of the  $\beta$ -chain of class-II antigens gave rise to a marked increase in the relative number of single positive (SP) T lymphocytes and their progenitors which were double positive for the thymocyte markers CD4 and CD8 antigens and these were coexpressed simultaneously. However, these phenomena were always accompanied by a significant decrease in the relative number of B-lymphocytes. These observations were not noted when the same blood samples were treated with



monoclonal antibodies to the homologous region of the  $\alpha$ -chain of class-II antigens or to the homologous region of class-I antigens.

5 Treatment of whole blood obtained from patients with B-cell chronic lymphocytic leukaemia (CLL) in a device according to the present invention with monoclonal antibody to the homologous region of the B chain of the HLA-DR antigen appeared to give rise to T-lymphopoiesis. This event was marked by the appearance of double positive cells coexpressing the CD4 and CD8 markers, the appearance of cells expressing CD34 and the concomitant increase in the number of single positive CD4<sup>+</sup> CD3<sup>+</sup> and  
10 CD8<sup>+</sup> CD3<sup>+</sup> lymphocytes. Furthermore, the immunophenotypic changes that took place in the generation of such cells were identical to those cited for thymocyte development, especially when measured with time.

The percentages of double positive cells (DP) generated at 2 hour incubation time of  
15 whole blood with monoclonal antibody to the homologous region of the  $\beta$ -chain of the DR antigen, decreased with time and these events were accompanied by increase in the percentages of single positive CD4<sup>+</sup> CD3<sup>+</sup> and CD8<sup>+</sup> CD3 cells simultaneously and at later times too. TCR  $\alpha$  and  $\beta$  chains were also expressed on these types of cells.

20 B-lymphocytes were constantly observed to lose markers such as CD19, CD21, CD23, IgM and DR and this coincided with the appearance of CD34<sup>+</sup> and CD34<sup>+</sup> CD2<sup>+</sup> cells, increases in CD7<sup>+</sup> cells, increases in CD8<sup>+</sup> CD28<sup>+</sup> and CD28<sup>+</sup> cells, increases in CD25<sup>+</sup> cells, the appearance of CD10<sup>+</sup> and CD34<sup>+</sup> cells and CD34<sup>+</sup> and CD19<sup>+</sup> cells increases in CD5<sup>+</sup> cells, and cells expressing low levels of CD45 antigen. These changes were due to  
25 treatment of blood with monoclonal antibody to the homologous region of the  $\beta$ -chain of HLA-DR antigen.

The immunophenotypic changes associated with such treatment is consistent with retrodifferentiation and subsequent commitment (i.e. recommitment) of B lymphocytes,  
30 because the majority of white blood cells in blood of patients with B-CLL before treatment were B lymphocytes. Furthermore, B-lymphocytes of patients with B-CLL which were induced to become T-lymphocytes following treatment with

cyclophosphamide and monoclonal antibody to the  $\beta$ -chain of HLA-DR antigen, were able to revert back to B lymphocytes following prolonged incubation with this treatment.

- On analysis of treated samples with monoclonal antibody to the  $\beta$ -chain of HLA-DR antigen, with CD16&56 and CD3 and CD8 and CD3 panels, the relative number of cells expressing these markers steadily increases in increments consistent with those determined with panels such as CD19 and CD3 and DR and CD3. Investigation of the supernatant of treated and untreated samples of patients with HIV infection using nephelometry and immunoelectrophoresis reveals increased levels of IgG indicating that the B-cells must have passed through the plasma cell stage. The increase in the relative number of all above-mentioned cells was also accompanied by the appearance of medium size heavily granulated cells expressing the CD56&16 antigens in extremely high amounts. Other cells which were extremely large and heavily granulated were observed transiently and these were positive for CD34 and double positive for CD4 CD8 markers. Other transient cells were also observed and these were large and granular and positive for the CD3 and CD19 receptors. CD25 which was present on the majority of B-lymphocytes was lost and became expressed by newly formed T-lymphocytes which were always observed to increase in number.
- CD28<sup>+</sup>CD8<sup>+</sup> and CD28<sup>+</sup> cells appeared after treatment of whole blood of patients with B-CLL with monoclonal antibody to the homologous region of the B chain of the DR antigen. These findings were due to treatment of blood with monoclonal antibody to the homologous region of the  $\beta$ -chain of HLA-DR antigen.
- T-lymphopoiesis generated in this manner was also observed in peripheral blood of healthy blood donors, cord blood, bone marrow, patients with various infections including HIV<sup>+</sup> individuals and AIDS patients, enriched fractions for B lymphocytes obtained from blood samples of healthy blood donors, IgA deficient patients and other patients with various other conditions. Furthermore, analysis of myeloid markers in treated samples of two patients with B-CLL with monoclonal antibody to the homologous region of the  $\beta$ -chain of the HLA-DR antigen showed a significant increase in the relative number of cells expressing the myeloid markers such as CD13 and CD33.

These markers were coexpressed with the CD56 & 16 or the CD7 antigens. However, the relative number of CD7<sup>+</sup> cells with T-lymphocyte markers and without myeloid antigens was observed on a separate population of cells. These particular observations were not seen in untreated samples or in samples treated with monoclonal antibodies to class I antigens or the homologous region of the  $\alpha$ -chain of HLA-DR antigen (see Charts 2 & 3). These final results suggest that B-lymphocytes once triggered via the  $\beta$ -chain of the HLA-DR antigen are not only able to regress into T lymphocyte progenitor cells but are also capable of existing into the myeloid and erythroid lineages.

Thus in summary, the data presented in the present application demonstrate that in a device according to the present invention (i) it is possible to convert healthy cells from one lineage to cells having the cell surface markers and morphological characteristics of cells of several other lineages and (ii) it is possible to obtain cells having the cell surface markers and morphological characteristics of primitive precursor cells (for example stem cells), from differentiated B lymphocytes and T lymphocytes.

It should be noted that a number of experiments have been carried out with BCLL cells. BCLL cells are mature B lymphocytes that are incapable of differentiating to the final terminally differentiated stage of a plasma cell. Instead, due to a chromosome defect, they exhibit high levels of proliferation, hence the large numbers of B lymphocytes in the blood of BCLL patients. By contrast to a number of tumour cells described in the prior art, BCLL cells have not undergone any form of limited reverse differentiation prior to use in the methods of the invention. Furthermore they do not exhibit any characteristics of undifferentiated cells in term of genomic structure, cell markers or cell morphology. They are in all respects mature B lymphocytes.

Thus whereas some malignant cells may have to a limited extent some characteristics of undifferentiated cells, this is not the case for BCLL cells, which are a perfectly acceptable experimental system for studying B lymphocytes. In fact BCLL and Daudi cells are not sufficiently distinguished from normal cells in any aspects relevant to these experiments. Indeed, the suitability of BCLL cells as a model system is

confirmed by Martensson *et al.*, 1989, Eur. J. Immunol. 19: 1625-1629 (see page 1625  
rhs, 1<sup>st</sup> para).

5 In addition, treatment of human buffy coat blood samples from healthy donors with  
CR3/43 monoclonal antibody resulted in a greater incidence of CD34 (stem cell  
marker) and a lower incidence of CD19 (B-lymphocyte marker).

10 It should be noted that the stem cells that are produced by the method of the present  
invention may be stem cells of any tissue and are not necessarily limited to  
lymphohaematopoietic progenitor cells.

Other modifications of the present invention will be apparent to those skilled in the art.

15 As will be readily apparent to those skilled in the art, the device of the present invention  
is not necessarily limited to use with a cell population including committed cells and/or  
an agent as disclosed herein, and is suitable for use with any procedure which requires the  
mixing and incubation of a fluid with an agent.

20 All publications mentioned in the above specification are herein incorporated by  
reference. Various modifications and variations of the described methods and systems of  
the invention will be apparent to those skilled in the art without departing from the scope  
and spirit of the invention. Although the invention has been described in connection with  
specific preferred embodiments, it should be understood that the invention as claimed  
25 should not be unduly limited to such specific embodiments. Indeed, various  
modifications of the described modes of carrying out the invention which are obvious to  
those skilled in molecular biology or related fields are intended to be within the scope of  
the following claims.

5

PATIENT ID	DIAGNOSIS	EXPT COND	WBC/L X10-9		%LYMPH		#LYMPH/L 10X-9		AGENT ML/mL
			B	A	B	A	B	A	
1	B-CLL	12HR AT 22C	100	ND	86.1	ND	86.1	ND	ANTI-B 50
2	B-CLL	2HR AT 22C	39.1	9.6	74.4	63.3	29.9	6.1	ANTI-B 50
		2HR AT 22C	39.1	37.7	74.4	75.1	29.9	28.3	ANTI-B PE 50
3	B-CLL	6HR AT 22C	39.5	9.3	71.9	67.2	28.3	6.2	ANTI-B 50
		6 HR AT 22C	39.5	37.7	71.9	72.5	28.3	27.4	ANTI-B PE 50
4	B-CLL	24HR AT 22C	39	9.3	73	66.5	28.4	6.2	ANTI-B 50
		24HR AT 22C	39	36.2	73	70.4	28.4	25.5	ANTI-B PE 50
5	B-CLL	2HR AT 22C							ANTI-B 50 ANTI-A 50 ANTI-I 50 ANTI-B & TOXIC AGENT 25+25

PATIENT ID	DIAGNOSIS	EXPT COND	WBC/L X10-9 B A	%LYMPH B A	#LYMPH/L 10X-9 B A	AGENT ML/mL
6	B-CLL	24HR AT 22C				ANTI-B 50
7	B-CLL	24HR AT 22C	170 128 178 130	95.4 91.1 94.2 90.4	16.9 11.6 16.8 11.9	ANTI-B 10 ANTI-I 10 ANTI-B & TOXIC AGENT 10+20
8	B-CLL	24HR AT 22C	16 7	81.9 51.2	14 3.0	ANTI-B 20
9	B-CLL	12HR AT 22C	+++ 89.5 +++ +++ 95.4	87 85.1 85.4 89.4 84.9	+++ 76.2 +++ +++	ANTI-B 30 ANTI-I 30 ANTI-4 30 ANTI- I+II+4 10+10+10
10	B-CLL	2HR AT 22C	19.3 ND	86 ND	16.7 ND	ANTI-B 30 ANTI-I 30
92	OUT PATIENT	2HR AT 22C	5.4 ND	74.5 ND	ND	ANTI-B 20
87	OUT PATIENT	2HR AT 22C	4.8 ND	59.3 ND	ND	ANTI-B 20
91	OUT PATIENT	2HR AT 22C	4.2 ND	54.0 ND	ND	ANTI-B 20
21	OUT PATIENT	2HR AT 22C	3.9 ND	47.4 ND	ND	ANTI-B 20

PATIENT ID	DIAGNOSIS	EXPT COND	WBC/L X10-9 B A	%LYMPH B A	#LYMPH/L 10X-9 B A	AGENT ML/mL
34	OUT PATIENT	2HR AT 22C	7.2 ND	20.0 ND	ND	ANTI-B 20
36	CMV INFANT	4HR AT 22C	13.4 ND	7.3 ND	ND	ANTI-B 20
93	HIV+ INFANT	4HR AT 22C	5.6 ND	43.4 ND	ND	ANTI-B 20
BB/ST	40% BLAST IN BLOOD 6 DAYS OLD	2HR AT 22C 24HR AT 22C	60.5 ND	20.2 ND	12.2 ND	ANTI-B 50 ANTI-A 50 ANTI-AB 25+25
HIV25	AIDS	2HR AT 22C	7.5 ND	34.8 ND	2.6 ND	ANTI-B 50 ANTI-A 50 ANTI-AB 25+25
43/BD	B CELL DEFICIENT	4HR AT 22C				ANTI-B 20 ANTI-I 20 ANTI-4 20
08/BD	B CELL DEFICIENT	4HR AT 22C				ANTI-B 20 ANTI-I 20 ANTI-4 20
PATIENT ID	DIAGNOSIS	EXPT COND	WBC/L X10-9 B A	%LYMPH B A	#LYMPH/L 10X-9 B A	AGENT ML/mL
HIV+	AIDS	6HR AT 22C				ANTI-B 20 ANTI-I
IgA-D	IgA DEFICIENT	6HR AT 22C				ANTI-B 20 ANTI-I 20

5 EXPT COND : EXPERIMENTAL CONDITIONS  
B : BEFORE

5

ANTI-A : monoclonal antibody to the homologous region of the  $\alpha$ -chain of HLA-DR antigen

ANTI-AB : both ANTI-B and ANTI-A added together

ANTI-4 : monoclonal antibody to the CD4 antigen

ANTI-I+II+4 : ANTI-I and ANTI-B and ANTI-4 added together

Cytotoxic agent : Cyclophosphamide

ML/ml : micro litre per ml

10

L : litre



TABLE 2

IMMUNOPHENOTYPING OF PATIENTS WITH B-CLL AND OTHER CONDITIONS  
BEFORE AND AFTER  
TREATMENT OF BLOOD SAMPLES WITH MONOCLONAL ANTIBODY  
TO THE HOMOLOGOUS REGION OF THE B CHAIN OF THE HLA-DR  
WITH CD19 AND CD3 MONOCLONAL ANTIBODIES.

PATIENT	% CD19+		% CD3+		%CD19+CD3+		%CD3-CD19-		% CD19+HG CD3- FC+	
	B	A	B	A	B	A	B	A	B	A
1	88	40	5	19	1	2	6	26	0	12
2	73	15	10	33	2	7	15	41	0	5
3	73	11	11	33	2	2	14	52	0	2
4	71	13	11	37	2	2	16	47	0	2
5	85	40	5	16	1	1	6	26	3	18
6	85	43	5	18	1	1	6	27	3	10
7	90	72	2	4	0	2	7	8	0	14
8	62	25	7	13	0	1	29	55	2	6
9	90	85	2	3	0	0	2	1	1	4
10	78	50	7	14	0	0	14	26	0	8
92	12	10	38	49	0	1	49	40	0	0
91	7	3	35	29	0	1	59	67	0	0
87	5	3	32	38	1	1	63	58	0	0
21	1	1	27	29	1	0	71	70	0	0
34	1	1	13	13	0	2	86	84	0	0
39	10	6	23	25	0	0	67	69	0	0
93	6	3	26	27	1	1	68	70	0	0
BB/ST	1	1	12	13	0	0	87	86	0	0
HIV25	7	2	26	27	0	0	68	67	0	0
43/BD	0	0	40	42	0	1	58	54	0	0
04/BD	0	0	49	41	0	3	43	41	0	0
HIV+	1	1	10	14	0	0	89	87	0	0
IgA/D	10	1	21	25	2	3	67	71	0	0

10 B: before treatment . A: after treatment

TABLE 3

IMMUNOPHENOTYPING OF PATIENTS WITH B-CLL  
AND OTHER CONDITIONS BEFORE AND AFTER TREATMENT OF BLOOD SAMPLES  
WITH MONOCLONAL ANTIBODY TO THE B CHAIN OF THE HOMOLOGOUS REGION  
OF THE HLA-DR WITH MONOCLONAL ANTIBODIES TO CD4 AND CD8.

PATIENT	%CD8+		%CD4+		%CD4+CD8+		%CD4-CD8-		CD4+LOW	
	B	A	B	A	B	A	B	A	B	A
1	2.8	16	2.9	11.4	0	3.2	93.1	67.6	0	0
2	6.2	13.2	9.1	24.3	0	9.4	78.7	46	5.8	6.3
3	7.2	13.1	7.4	23.9	0	8.2	78.8	48.1	6.3	6.6
4	10.1	24.2	7.6	24.9	0.3	2.8	77.5	42	4.6	5
5	2.9	16.2	1.8	7.6	0	2	95	62.3	0	0
6	ND	12	ND	8.1	ND	1.7	ND	75.7	ND	0
7	1.9	2.6	1.9	2.8	0	0	95.8	94.3	0	0
8	3.2	7	3.9	6.9	0.1	2	87.3	79.8	4.3	6
9	2.8	2.9	3	3	0	0	94	94.1	0	0
10	5.7	9.4	4.7	9.1	0.6	0.8	88.7	79.2	0	0
92	21	19	21.6	21	0.8	1.9	50.5	52.5	5.3	4.8
91	15.4	18.1	13.6	17.9	6.2	2.6	57	57.3	7.3	3.5
87	16.8	21.8	13.4	20.4	2.9	2.6	59.5	48.9	7	5.6
21	16	24.1	9.1	15.2	1	2.6	69.6	53.2	3.7	4.2
34	9.4	11.9	5.7	4.9	2	3.3	67.6	65.3	14.4	14.5
39	12.1	12.6	13.1	14.6	0.4	1.3	62.3	66.7	11.9	4.3
93	18.9	20.3	9.7	10.3	1.8	1.4	65.5	65.9	3.4	1.8
BB/ST	6.3	13	5.7	7.3	2.2	1.1	34.7	70.3	50.3	7.6
HIV25	24.1	24.9	0.8	1.1	1.3	5	70.2	69.3	2.9	3.8

**TABLE 4**

**IMMUNOPHENOTYPING OF PATIENTS WITH B-CLL AND OTHER CONDITIONS  
BEFORE AND AFTER TREATMENT OF SAMPLES WITH MONOCLONAL ANTIBODY TO  
THE B CHAIN OF THE HLA-DR WITH MONOCLONAL ANTIBODIES TO CD3 AND DR**

PATIENT	DR+		CD+		CD+DR+		DR-CD3-		DR+HCD3-	
	B	A	B	A	B	A	B	A	B	A
1	87	45.5	3.5	20.8	2.5	4.2	6.9	21.6	0	7.6
2	76.2	19.4	9.6	29.2	3.9	8.7	10.3	36.8	0	5.5
3	77.7	18.3	8.4	29.4	4.1	8.8	9.6	38.1	0	4.7
4	76.8	19.2	7.6	29.5	6.2	10.5	9.1	37.2	0	3.3
5	ND	47.1	ND	11.5	ND	9.9	ND	22.4	ND	7.3
6	ND									
7	91.4	85.8	2.4	2.5	0.7	0.7	5.1	4.2	0	6.3
8	61.8	28.9	6.5	11.2	2	3.3	28.6	54.6	0	1.5
9	ND									
10	82.6	44.7	4.3	9.8	3.3	5	9.8	22.2	0	17.9
92	23.8	14.1	39.3	41.9	4.5	3.5	32.4	40.5	0	0
91	13.3	7.9	29.6	32.5	3.4	2.9	53.4	56.5	0	0
87	14.8	12.2	28.4	34.1	5.5	6.6	51.1	46.5	0	0
21	ND									
34	11.9	12.9	10.4	13.7	0.8	0.6	76.7	72.8	0	0
39	25.6	13.7	24.6	25.2	3	2.8	46.5	25.2	0	0
93	13.3	8.9	18.4	18.9	9.9	10.1	58.2	61.7	0	0
BB/ST	44.2	32.5	11.7	12.2	0.8	0.8	43	49.4	0	4.6

**TABLE 5**

IMMUNOPHENOTYPING OF PATIENTS WITH B-CLL AND OTHER CONDITIONS BEFORE AND AFTER TREATMENT OF BLOOD SAMPLES WITH MONOCLONAL ANTIBODY TO THE HOMOLOGOUS REGION OF THE B CHAIN OF THE HLA-DR WITH MONOCLONAL ANTIBODIES TO CD16+56 AND CD3.

PATIENTS	CD56+&16		CD3+		CD56+&16+C D3+		CD56+&16-CD3-	
	B	A	B	A	B	A	B	A
1	2	4.3	5.7	19.7	0.7	1.7	91.3	73
2	11.5	38.9	12.4	32.6	1	6.6	74.5	21
3	12	36.2	12.1	34.5	0.7	6	75.5	23
4	12.2	32.6	12.4	39.6	0.5	5	74.7	22.2
5	ND	13.1	ND	9.4	ND	2.6	ND	73.5
6	ND							
7	0.8	0.8	2.8	2.4	0.3	0.2	96.2	96.4
8	24.8	52	5.4	12.4	0.9	4.1	68.3	31.1
9	ND							
10	1.1	1.3	6.1	13.7	2.1	2.5	90.5	82.4
92	23.8	34.5	44.3	44.8	2	1.5	29.2	18.6
91	4.6	3.9	28.8	29.4	3	3.2	63.3	63.3
87	47.9	46.4	28.8	36.5	5.8	3.7	16.9	13
21	9.4	9.4	19.7	23.6	4.2	6.7	66	59.5

PATIENTS	CD56+&16		CD3+		CD56+&16+C D3+		CD56+&16-CD3-	
34	21.5	12.8	11.4	13.7	1.8	0.6	64.6	72.8
39	7	2.7	23.4	26.1	1.1	0.1	68.2	71
93	55.8	54.9	26.2	26.3	1.7	2	16.1	16.8
BB/ST	28.8	29.9	12	14.3	0.8	1.8	49.4	53.6

TABLE 6

IMMUNOPHENTYPING OF PATIENTS WITH B-CLL AND OTHER CONDITIONS BEFORE  
AND AFTER OF TREATMENT OF BLOOD WITH MONOCLONAL ANTIBODY TO THE  
HOMOLOGOUS REGION OF THE B CHAIN OF THE HLA-DR WITH MONOCLONAL  
ANTIBODIES TO CD45 AND CD14.

PATIENTS	CD45+H		CD45+L		CD45+CD14+	
	B	A	B	A	B	A
1	90.5	70.1	7.5	21.9	0.8	3.3
2	85.8	52.2	8.8	38.3	5.3	9.5
3	84.3	52.2	9.9	33.8	5.1	13.2
4	91.5	79.2	2.1	7	5.7	10.8
5	63.1	84.6	34.9	9.4	0.5	3.6
6	ND					
7	52.8	85.2	45.6	13.9	0.5	0.6
8	71.1	55	71.1	34.5	5.3	8.7
9	SEE					
10	79.7	47.3	16.3	48	2.1	1.9
92	61.7	64.7	27.4	26.6	5.9	3.6
91	49.4	49.2	40.4	44.3	6.5	3.2
87	52.4	61.5	36.1	28.7	7	6.5
21	45.8	43.3	44.3	47.6	6.2	3.3
34	24.4	24.6	54.8	59.6	13.3	9.7
39	48.7	46.3	30.5	42.1	14.5	8.8
93	SEE					
HIV+	22.6	26.9	66.8	63.5	6.8	6.7
IgA/D	47.4	59.8	41.9	33.3	5.9	4.1

**TABLE 7**

IMMUNOPHENOTYPING OF PATIENT WITH B-CLL AND OTHER CONDITIONS BEFORE AND AFTER TREATMENT OF BLOOD WITH MONOCLONAL ANTIBODIES TO THE HOMOLOGOUS REGION OF THE B-CHAIN OF THE HLA-DR WITH MONOCLONAL ANTIBODIES TO CD8 AND CD3.

PATIENTS	CD8+		CD3+		CD8+CD 3+		CD8- CD3-	
	B	A	B	A	B	A	B	A
2	0.6	1.3	7.5	19.3	4.2	19.3	87.7	63.8
3	1.1	1.4	8.3	20.3	5.6	18.4	84.8	59.8
4	3.5	2.9	8.3	27	3.9	16.6	84.2	53.1
92	3.5	1.9	27.6	25.2	18.4	19	50.3	52.8
91	4	3.1	18.2	19	14.1	12.6	63.6	65.3
87	5.7	3.9	19.9	23.6	15.4	17.4	58.8	55
21	4.8	7.4	16.3	17.3	13.7	13	65.2	62
34	3	3.6	5.2	6.7	7.6	7.5	84.1	82.3

**TABLE 8**

IMMUNOPHENOTYPING OF A PATIENT WITH B-CLL WITH TIME AFTER TREATMENT OF BLOOD WITH PE CONJUGATED MONOCLONAL ANTIBODY TO THE HOMOLOGOUS REGION OF THE B-CHAIN OF THE HLA-DR MEASURE WITH MONOCLONAL ANTIBODIES TO CD45 AND CD14.

TIME	DR+CD45+CD14+r	CD45+L	CD45+H
2HR	81.7	8.2	8.2
6HR	80.7	8.1	10.6
24HR	79	1.1	18.4

**TABLE 9**

IMMUNOPHENOTYPING OF A PATIENT WITH B-CLL WITH TIME AFTER TREATMENT OF BLOOD WITH PE CONJUGATED MONOCLONAL ANTIBODY TO THE HOMOLOGOUS REGION OF THE B-CHAIN OF THE HLA-DR MEASURED WITH MONOCLONAL ANTIBODIES TO CD19 AND CD3.

TIME	CD19+DR+r	CD3+	CD3+DR+	CD19-CD3-DR-
2HR	87.4	10.1	1.8	10.7
6HR	75.5	10.4	3.1	10.7
24HR	74	11.7	2.9	11

**TABLE 10**

IMMUNOPHENOTYPING OF A PATIENT WITH B-CLL WITH TIME AFTER TREATMENT OF BLOOD WITH PE CONJUGATED MONOCLONAL ANTIBODY TO THE HOMOLOGOUS REGION OF THE B-CHAIN OF THE HLA-DR MEASURED WITH MONOCLONAL ANTIBODIES TO CD4 AND CD8.

TIME	CD8+& DR+r	CD4+	CD4+&CD8+&DR+r	CD4+DR+	CD4-CD8-DR-
2HR	77.6	6.8	5.4	1.3	8.8
6HR	75.8	6.7	6.4	1.8	9.3
24HR	77	6.4	4.8	1.9	11

**TABLE 11**

IMMUNOPHENOTYPING OF A PATIENT WITH B-CLL WITH TIME AFTER TREATMENT OF BLOOD WITH PE CONJUGATED MONOCLONAL ANTIBODY TO THE HOMOLOGOUS REGION OF THE B-CHAIN OF THE HLA-DR MEASURED WITH MONOCLONAL ANTIBODIES TO CD3 AND DR.

TIME	DR+	CD3+	CD3+DR+	CD3+DR-
2HR	75	9.5	4.2	10.9
6HR	74.8	8.8	4.8	10.9
24HR	ND	ND	ND	ND



**TABLE 12**

5 IMMUNOPHENOTYPING OF A PATIENT WITH B-CLL WITH TIME AFTER TREATMENT  
OF BLOOD WITH PE CONJUGATED MONOCLONAL ANTIBODY TO THE HOMOLOGOUS  
REGION OF THE B-CHAIN OF THE HLA-DR MEASURED WITH MONOCLONAL  
ANTIBODIES TO CD16&56 AND CD3.

TIME	CD56+&16+DR+r	CD3+	CD56+CD16+&CD3 +DR+r	CD56-CD16- &CD16-DR-
2HR	82.5	9.5	4.1	3.5
6HR	84.3	7.5	4.1	3.3
24HR	ND	ND	ND	ND

**TABLE 13**

10 IMMUNOPHENOTYPING OF A PATIENT WITH B-CLL WITH TIME AFTER TREATMENT  
OF BLOOD WITH PE CONJUGATED MONOCLONAL ANTIBODY TO THE HOMOLOGOUS  
REGION OF THE B-CHAIN OF THE HLA-DR MEASURED WITH MONOCLONAL  
15 ANTIBODIES TO CD8 AND CD3.

TIME	CD8+DR+	CD3+	CD8+CD+3&DR+r	CD8-CD3-DR-
2HR	76.2	6.6	6.7	10.6
6HR	76.5	6.2	6.2	10.3

TABLE 14

5 IMMUNOPHENOTYPING OF PATIENTS WITH B-CLL BEFORE AND AFTER TREATMENT  
OF BLOOD WITH MONOCLONAL ANTIBODIES TO THE HOMOLOGOUS REGION OF  
THE A-CHAIN OF THE HLA-DR, THE HOMOLOGOUS REGION OF THE B-CHAIN OF THE  
HLA-DR , THE TWO MONOCLONAL TOGETHER, MONOCLONAL TO THE  
HOMOLOGOUS REGION OF THE B-CHAIN PLUS CYCLOPHOSPHOAMIDE AND THE  
HOMOLOGOUS REGION OF CLASS I ANTIGENS MEASURED WITH TIME.

10

	CD19+					CD3+					CD19+CD3+					CD19-CD3-				
ID	B	A	AB	A	AI	B	A	AB	A	AI	B	A	AB	A	AI	B	A	AB	A	AI
		A		BC			A		BC			A		BC			A		BC	
5/6																				
2H	86	91	54	40	89	5	4	16	23	5	1	1	3	2	1	6	4	27	33	5
24	N	88	51	60	86	N	4	18	10	4	N	2	1	2	3	N	4	29	28	7
10																				
2H	77	N	59	N	80	7	N	13	N	7	1	N	1	N	0	14	N	26	N	12
09																				
24	8	N	N	N	6	32	N	N	N	38	1	N	N	N	1	59	N	N	N	56
43/ BD																				
6H	0	N	0	0	0	40	N	42	43	49	0	N	1	0	1	58	N	54	54	47
04/ BD																				
6H	0	N	0	0	0	49	N	41	45	46	0	N	3	1	3	43	N	42	44	41
HIV +																				
6H	1	N	0	N	1	10	N	14	N	12	0	N	0	N	0	89	N	86	N	87
IgA /D																				
6H	10	N	1	N	12	21	N	25	N	20	2	N	1	N	3	67	N	71	N	68

15 B = Before; A = After; AB = after addition to antibody to beta chain; AA = after addition of  
antibody to alpha chain; ABC = after addition of antibody to either alpha or beta chain and  
cyclophosphamide; A1 = after addition of antibody to Class I.

## CD8 AND CD4

10

	CD8+					CD4+					CD4+CD8+					CD4-CD8-				
ID	B	A	AB	A	AI	B	A	AB	A	AI	B	A	AB	A	AI	B	A	AB	A	AI
		A		BC			A		BC			A		BC			A		BC	
5/6																				
2H	3	2	14	10	4	2	2	8	8	3	0	0	3	2	1	95	94	74	79	93
24	N	3	9	4	4	N	3	8	4	3	N	0	2	2	0	N	94	81	90	93
10																				
2H	3	N	7	N	4	4	N	7	N	3	1	N	2	N	1	91	N	83	N	92
09																				
24	10	N	N	N	15	21	N	N	N	38	2	N	N	N	2	61	N	N	N	53

### CD3 AND DR

10

	DR+					CD3+					CD3+DR+					CD3-DR-				
ID	B	A	AB	A	AI	B	A	AB	A	AI	B	A	AB	A	AI	B	A	AB	A	AI
		A		BC			A		BC			A		BC			A		BC	
5/6																				
2H	N	90	54	N	87	N	4	12	N	4	N	2	10	N	3	N	5	22	N	5
10																				
2H	83	N	63	N	81	4	N	8	N	4	4	N	7	N	4	9	N	23	N	12
09																				
24	14	N	N	N	13	30	N	N	N	36	3	N	N	N	3	51	N	N	N	47

15

**TABLE 17****CD16&56 AND CD3**

	CD56+&16+					CD3+					CD56+&16+CD3+					CD56-&16-CD3-				
ID	B	A	AB	A	AI	B	A	AB	A	AI	B	A	AB	A	AI	B	A	AB	A	AI
		A		BC			A		BC			A		BC			A		BC	
5/6																				
2H	N	0	13	N	4	N	5	9	N	5	N	1	3	N	1	N	94	74	N	90
10																				
2H	0	N	1	N	1	6	N	14	N	6	1	N	2	N	1	92	N	65	N	92
09																				
24	42	N	N	N	41	36	N	N	N	38	2	N	N	N	2	20	N	N	N	19

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**TABLE 18****CD45 AND CD14**

	CD45+L					CD45+M					CD45+H					CD45+CD14+				
ID	B	A	AB	A	AI	B	A	AB	A	AI	B	A	AB	A	AI	B	A	AB	A	AI
		A		BC			A		BC			A		BC			A		BC	
5/6																				
2H	0	0	5	10	0	44	43	50	50	32	55	43	50	31	67	1	1	1	2	0
10																				
2H	0	N	0	N	0	43	N	54	N	35	54	N	42	N	62	1	N	1	N	0
09																				
24	2	N	N	N	1	18	N	N	N	16	71	N	N	N	76	7	N	N	N	5
HIV+																				
6H	4	N	3	N	6	63	N	61	N	41	23	N	27	N	40	7	N	7	N	7
IgA/D																				
6H	2	N	2	N	4	40	N	31	N	44	47	N	60	N	44	6	N	4	N	6

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**TABLE 19****CD8 AND CD28**

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	CD8+					CD28+					CD8+CD28+					CD8-CD28-				
ID	B	A	AB	A	AI	B	A	AB	A	AI	B	A	AB	A	AI	B	A	AB	A	AI
		A		BC			A		BC			A		BC			A		BC	
5/6																				
2H	N	3	6	N	3	N	1	4	N	2	N	1	4	N	1	N	95	86	N	94
8																				
2H	4	N	6	N	N	3	N	5	N	N	1	N	3	N	N	92	N	86	N	N

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**TABLE 20****CD34 AND CD2**

	CD34+					CD2+					CD34+CD2+					CD34-CD2-				
ID	B	A	AB	A	AI	B	A	AB	A	AI	B	A	AB	A	AI	B	A	AB	A	AI
		A		BC			A		BC			A		BC			A		BC	
5/6																				
2H	N	1	34	N	N	N	6	13	N	N	N	3	30	N	N	N	90	21	N	N
24	N	1	6	9	N	N	7	23	4	N	N	3	33	43	N	N	87	34	34	N
HIV+																				
2H	2	1	12	13	N	20	21	21	12	N	4	5	9	14	N	73	73	64	60	N
BB/ST																				
2H	26	23	33	14	N	15	14	15	15	N	3	30	23	36	N	27	32	28	35	N
											1									
24	N	11	29	11	N	N	13	12	9	N	N	27	9	18	N	N	48	49	61	N

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TABLE 21

FACs analysis using human buffy coat derived cells

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CD Marker	Initial Analysis	2 h analysis Untreated cells	2 h analysis Treated cells	24 h analysis Untreated cells	24 h analysis Treated cells	5 d analysis Untreated cells	5d analysis Treated cells
CD 45							
CD 38	54.9	55.6	30.1	51.8	52.8	13.0	5.4
CD34	0.6	0.5	11.5	9.7	8.0	0.4	25.1
CD10	0.2	0.0	0.9	1.9	1.8	0.0	0.2
CD19	13.2	0.0	15.1	11.1	2.3	8.7	0.7
CD34	1.8	13.1	0.4	13.0	9.1	0.4	25.8
CD3	56.3	62.6	29.7	46.9	69.2	79.8	63.4
CD117	0.3	0.6	0.0	1.3	0.8	0.2	0.1
CD34	1.8	0.6	7.4	6.4	6.0	0.2	24.6
CD71	1.5	0.4	0.0	4.8	3.9	0.2	42.9
GLYCA	5.4	2.3	10.1	3.6	2.8	3.8	1.8
CD34	1.3	0.6	14.3	12.5	8.9	0.4	24.3
CD90	18.4	12.6	0.0	9.3	5.0	0.1	0.3
AC133	0.1	0.0	2.0	0.3	0.4	0.0	0.1
CD34	1.5	0.6	11.9	10.8	7.7	0.0	13.2

Values are expressed as the percentage of cells expressing markers

CHART 1

5 IMMUNOPHENOTYPIC CHANGES OF UNTREATED AND TREATED BLOOD SAMPLE OF  
 PATIENT (2, 3 & 4) WITH MONOCLONAL ANTIBODY TO THE HOMOLOGOUS REGION  
 OF THE  $\beta$ -CHAIN OF HLA-DR ANTIGEN MEASURED WITH TIME.

WITHOUT	WITH	FL1	FL2	TIME
NOTHING001	WITH002	CD45	CD14	2HR
NO001	WE002	CD45	CD14	6HR
001001	002002	CD45	CD14	24HR
NOTHING003	WITH004	CD3	CD19	2HR
NO003	WE004	CD3	CD19	6HR
001003	002004	CD3	CD19	24HR
NOTHING004	WITH005	CD4	CD8	2HR
NO004	WE005	CD4	CD8	6HR
001004	002005	CD4	CD8	24HR
NOTHING005	WITH006	CD3	DR	2HR
NO005	WE006	CD3	DR	6HR
001005	002006	CD3	DR	24HR
NOTHING006	WITH007	CD3	CD56&16	2HR
NO006	WE007	CD3	CD56&16	6HR
001006	002007	CD3	CD56&16	24HR
N003	W004	CD3	CD8	2HR
NO007	WE008	CD3	CD8	6HR
001007	002008	CD3	CD8	24HR

## CHART 1A

5 IMMUNOPHENOTYPIC CHANGES OF UNTREATED AND TREATED BLOOD SAMPLE OF PATIENT (2, 3, 4) WITH MONOCLONAL ANTIBODY TO THE HOMOLOGOUS REGION OF THE  $\beta$ -CHAIN OF HLA-DR ANTIGEN CONJUGATED TO PE MEASURED WITH TIME.

ID	FL1	FL2	TIME
WL003	CD45	CD14	2HR
WEL003	CD45	CD14	6HR
003003	CD45	CD14	24HR
WL005	CD3	CD19	2HR
WEL005	CD3	CD19	6HR
003005	CD3	CD19	24HR
WL006	CD4	CD8	2HR
WEL006	CD4	CD8	6HR
003006	CD4	CD8	24HR
WL007	CD3	DR	2HR
WEL 007	CD3	DR	6HR
WL008	CD3	CD65&16	2HR
WEL 008	CD3	CD56&16	6HR
WL005	CD3	CD8	2HR
WEL009	CD3	CD8	6HR



## 5

**2016年12月**

WITH	WITHOUT	FL1	FL2	TIME
H2B001: AB		CD7	CD33&13	2HR
I2A001: AA		CD21	CD5	2HR
I2B001:AB		CD21	CD5	2HR
J2A001: AA		CD34	CD2	2HR
J2B001: AB		CD34	CD2	2HR
B2A24H001:AA		CD34	CD2	24HR
B2B24H001:AB		CD34	CD2	24HR
B2BX24H001: ABC		CD34	CD2	24HR
K2B001: AB		CD10	CD25	2HR
K2A001: AA		CD10	CD25	2HR

**IMMUNOPHENOTYPIC CHANGES OF UNTREATED AND TREATED BLOOD OF PATIENT (8) WITH MONOCLONAL ANTIBODY TO THE HOMOLOGOUS REGION OF THE  $\beta$ -CHAIN OF HLA-DR ANTIGEN.**

WITH	WITHOUT	FL1	FL2	TIME
A2001	AN001	CD45	CD14	2HR
		CD45	CD14	2HR
C2001	CN001	CD3	CD19	2HR
		CD3	CD19	2HR
D2001	DN001	CD4	CD8	2HR
		CD4	CD8	2HR
E2001	EN001	CD3	DR	2HR
		CD3	DR	2HR
F2001	FN001	CD3	CD56&16	2HR
		CD3	CD56&16	2HR
G2001	GN001	CD28	CD8	2HR
		CD28	CD8	2HR
H2001	HN001	CD7	CD5	2HR
		CD7	CD5	2HR
I2001	IN001	CD13	CD20	2HR
		CD13	CD20	2HR
J2001	JN001	CD45RA	CD25	2HR
		CD45RA	CD25	2HR
K2001	KN001	CD57	CD23	2HR
		CD57	CD23	2HR

## 5

WITH	WITHOUT	FL1	FL2	TIME
	CLL0001	CD45	CD14	2HR
CLL1001		CD45	CD14	2HR
CLL2001		CD45	CD14	2HR
	CLL0003	CD3	CD19	2HR
		CD3	CD19	2HR
CLL1003		CD3	CD19	2HR
CLL2003		CD3	CD19	2HR
	CLL0004	CD4	CD8	2HR
CLL1004		CD4	CD8	2HR
CLL2004		CD4	CD8	2HR
	CLL005	CD3	DR	2HR
CLL1005		CD3	DR	2HR
CLL2005		CD3	DR	2HR
	CLL0006	CD3	CD56&16	2HR
CLL1006		CD3	CD56&16	2HR
CLL2006		CD3	CD56&16	2HR

The invention is further described by the following numbered paragraphs:

1. A device for forming and/or increasing the relative number of undifferentiated cells in a cell population including committed cells, which device comprises a chamber, means for introducing into said chamber a cell population including committed cells, means for introducing into said chamber retrodifferentiation means that are capable of causing a committed cell to retrodifferentiate into an undifferentiated cell, and incubation means for incubating said committed cells in the presence of said retrodifferentiation means such that a committed cell retrodifferentiates into an undifferentiated cell.
2. A device for forming and/or increasing the relative number of undifferentiated cells in a cell population including committed cells, which device comprises a chamber, means for introducing into said chamber a cell population including committed cells, means for introducing into said chamber an agent that causes the committed cell to retrodifferentiate into an undifferentiated cell, and incubation means for incubating said agent and said committed cells such that a committed cell retrodifferentiates into an undifferentiated cell.
3. A device according to paragraphs 1 or 2 wherein said device comprises measuring means for measuring the volume of said cell population.
4. A device according to paragraphs 1-3 wherein said device comprises means for conducting cell counts and for measuring the cell concentration of said cell population.
5. A device according to paragraph 4 wherein the means for conducting cell counts is a coulter counter, preferably miniaturised, or other suitable cytometer.
6. A device according to any one of the preceding paragraphs wherein said device comprises transfer means for transferring an amount of said cell population from a storage container to said chamber.
7. A device according to any one of the preceding paragraphs wherein said device comprises transfer means for transferring a pre-determined amount of said cell population from a storage container to said chamber.
8. A device according to any one of the preceding wherein said device comprises calculator means for calculating the volume of agent to be added to the chamber.

9. A device according to any one of the preceding paragraphs wherein said device comprises a further transfer means for transferring a volume of agent to the chamber, preferably said further transfer means is a syringe driven by a motor.
10. A device according to any one of the preceding paragraphs wherein said device comprises a further transfer means for transferring a calculated volume of agent to the chamber.
11. A device according to paragraphs 9 or 10 wherein said further transfer means is a syringe driven by a motor.
12. A device according to any one of the preceding paragraphs wherein said device comprises carbon dioxide control means for controlling the concentration of carbon dioxide in said chamber.
13. A device according to any one of the preceding paragraphs wherein said device comprises temperature control means for controlling the temperature in said chamber.
14. A device according to any one of the preceding paragraphs wherein said device comprises mixing means for mixing the cell population and agent within the chamber.
15. A device according to any one of the preceding paragraphs wherein said device comprises timing means for timing the incubation period.
16. A device according to any one of the preceding paragraphs wherein said device comprises display means for displaying to the user the remaining time period of the incubation period.
17. A device according to any one of the preceding paragraphs wherein said device comprises alarm means for alerting the user of completion of the incubation period.
18. A device according to any one of the preceding paragraphs wherein said device comprises harvesting means for harvesting cells from the chamber.
19. A device according to paragraph 18 wherein the harvesting means harvests the undifferentiated cells from the chamber.
20. A device according to any one of the preceding paragraphs wherein said device comprises removal means for removing a sample of cells, comprising undifferentiated cells, from the chamber into a storage container.

21. A device according to any one of the preceding paragraphs wherein said device comprises sealing means for sealing a storage container comprising a population of cells comprising undifferentiated cells.
22. A device according to any one of the preceding paragraphs wherein the committed cells are non-cancer cells.
23. A device according to any one of the preceding paragraphs wherein the committed cells are differentiated cells.
24. A device according to any one of the preceding paragraphs wherein the committed cells are committed haematopoietic cells.
25. A device according to any one of the preceding paragraphs wherein the committed cells are selected from CFC-T cells, CFC-B cells, CFC-Eosin cells, CFC-Bas cells, CFC-GM cells, CFC-MEG cells, CFC-E cells, T cells and B cells.
26. A device according to any one of the preceding paragraphs wherein the undifferentiated cells are pluripotent stem cells.
27. A device according to any one of the preceding paragraphs wherein the undifferentiated cells are stem cells selected from the group consisting of haematopoietic stem cells, neuronal stem cells, epithelial stem cells, mesenchymal stem cells, endodermal stem cells and embryonic stem cells.
28. A device according to any one of the preceding paragraphs wherein the undifferentiated cells are characterised by one or more of the following cell surface marker designations: CD34<sup>+</sup>, HLA-DR<sup>+</sup>, CD38<sup>+</sup>, CD117, AC133, CD90 and/or CD45<sup>low</sup>.
29. A device according to any one of the preceding paragraphs wherein the undifferentiated cells are MHC class I<sup>+</sup> and/or MHC class II<sup>+</sup> cells.
30. A device according to any one of the preceding paragraphs wherein the agent engages a receptor that mediates capture, recognition or presentation of an antigen at the surface of the committed cells.
31. A device according to paragraph 30 wherein the receptor is an MHC class I antigen or an MHC class II antigen.
32. A device according to paragraph 31 wherein the class I antigen is an HLA-A receptor, an HLA-B receptor, an HLA-C receptor, an HLA-E receptor, an HLA-F

receptor or an HLA-G receptor and said class II antigen is an HLA-DM receptor, an HLA-DP receptor, an HLA-DQ receptor or and HLA-DR receptor.

33. A device according to paragraph 32 wherein the receptor is an HLA-DR receptor.

34. A device according to paragraph 30 wherein the receptor comprises a  $\beta$ -chain having homologous regions.

35. A device according to paragraph 34 wherein the receptor comprises at least the homologous regions of the  $\beta$ -chain of HLA-DR.

36. A device according to paragraph 30 wherein the agent is an antibody to the receptor.

37. A device according to paragraph 36 wherein the agent is a monoclonal antibody to the receptor.

38. A device according to paragraphs 36 or 37 wherein the antibody is selected from the group consisting of monoclonal antibody CR3/43 and the monoclonal antibody TAL 1B5.

39. A device according to any one of paragraphs 2-38 wherein the agent modulates MHC gene expression.

40. A device according to paragraph 39 wherein the agent modulates MHC class I<sup>+</sup> and/or MHC class II<sup>+</sup> expression.

41. A device according to any one of the preceding paragraph wherein the cell population including committed cells is a buffy coat blood sample or is from a buffy coat blood sample.

42. A device for forming and/or increasing the relative number of undifferentiated cells in a cell population including haematopoietic cells, which device comprises a chamber, means for introducing into said chamber a cell population including haematopoietic cells, means for introducing into said chamber retrodifferentiation means that are capable of causing a committed haematopoietic cell to retrodifferentiate into an undifferentiated cells, and incubation means for incubating said committed cells in the presence of said retrodifferentiation means such that a committed haematopoietic cell retrodifferentiates into an undifferentiated cell.

43. A device for forming and/or increasing the relative number of cells having a cell surface marker designation CD34<sup>+</sup> and/or HLA-DR<sup>+</sup> and/or CD38<sup>+</sup> and/or CD117 and/or

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44. A method of preparing an undifferentiated cell, the method comprising retrodifferentiating a more committed cell to an undifferentiated cell, wherein the retrodifferentiation of the more committed cell occurs to the more committed cell in or from a buffy coat blood sample.

46. A method according to paragraphs 44-45 wherein the committed cells are non-cancer cells.

48. A method according to any one of paragraphs 44-47 wherein the committed cells are committed haematopoietic cells:

50. A method according to any one of paragraphs 44-49 wherein the undifferentiated cells are pluripotent stem cells.

51. A method according to any one of paragraphs 44-50 wherein the undifferentiated cells are stem cells selected from the group consisting of haematopoietic stem cells, neuronal stem cells, epithelial stem cells, mesenchymal stem cells and embryonic stem cells.

52. A method according to any one of the preceding paragraphs wherein the undifferentiated cells are characterised by one or more of the following cell surface marker designations: CD34<sup>+</sup>, HLA-DR<sup>+</sup>, CD38<sup>+</sup>, CD117, AC133, CD90 and/or CD45low.
53. A method according to any one of paragraphs 44-52 wherein the undifferentiated cells are MHC class I<sup>+</sup> and/or MHC class II<sup>+</sup> cells.
54. A method according to any one of paragraphs 44-53 wherein the agent engages a receptor that mediates capture, recognition or presentation of an antigen at the surface of the committed cells.
55. A method according to paragraph 54 wherein the receptor is an MHC class I antigen or an MHC class II antigen.
56. A method according to paragraph 55 wherein the class I antigen is an HLA-A receptor, an HLA-B receptor, an HLA-C receptor, an HLA-E receptor, an HLA-F receptor or an HLA-G receptor and said class II antigen is an HLA-DM receptor, an HLA-DP receptor, an HLA-DQ receptor or and HLA-DR receptor.
57. A method according to paragraph 56 wherein the receptor is an HLA-DR receptor.
58. A method according to paragraph 54 wherein the receptor comprises a  $\beta$ -chain having homologous regions.
59. A method according to paragraph 58 wherein the receptor comprises at least the homologous regions of the  $\beta$ -chain of HLA-DR.
60. A method according to paragraph 54 wherein the agent is an antibody to the receptor.
61. A method according to paragraph 60 wherein the agent is a monoclonal antibody to the receptor.
62. A method according to paragraphs 60 or 61 wherein the antibody is selected from the group consisting of monoclonal antibody CR3/43 and the monoclonal antibody TAL 1B5.
63. A method according to any one of paragraphs 45-62 wherein the agent modulates MHC gene expression.
64. A method according to paragraph 63 wherein the agent modulates MHC class I<sup>+</sup> and/or MHC class II<sup>+</sup> expression.

65. A method of increasing the relative number of cells having a cell surface marker designation CD34<sup>+</sup> and/or HLA-DR<sup>+</sup> and/or CD38<sup>+</sup> and/or CD117 and/or AC133 and/or CD90 and/or CD45<sup>low</sup> in a buffy coat blood sample, the method comprising contacting a more committed cell in a buffy coat blood sample with an agent that operably engages said committed cells, such that the relative number of CD34<sup>+</sup> and/or HLA-DR<sup>+</sup> and/or CD38<sup>+</sup> and/or CD117 and/or AC133 and/or CD90 and/or CD45<sup>low</sup> cells increases as a result of said engaging.
66. A method of preparing an undifferentiated cell, the method comprising contacting one or more differentiated cells in a cell population with retrodifferentiation means effective to displace the ratio of normal differentiated cells in said population, whereby one or more of said differentiated cells is caused to retrodifferentiate to an undifferentiated cell(s).
67. Use of retrodifferentiating means to displace the ratio of normal differentiated cells in a cell population to effect retrodifferentiation of one or more of said differentiated cells to an undifferentiated cell(s).
68. A method of preparing an undifferentiated cell, the method comprising retrodifferentiating a differentiated cell in a cell population to an undifferentiated cell, wherein the environment comprising said cell population comprising one or more differentiated cells is changed from a first environment to a second environment wherein the free ion concentration of said second environment is effectively modified as compared with the first environment so as to cause one or more of said differentiated cells to retrodifferentiate to an undifferentiated cell(s).
69. A method of preparing an undifferentiated cell, the method comprising contacting one or more differentiated cells in a cell population with retrodifferentiation means effective to displace the ratio of normal differentiated cells, culturing the cell population in a ion free or ion sequestered first environment, and changing the first environment to a second environment wherein the concentration of ions present in the second environment is effectively modified as compared with the first environment, thus to effect one or more of the differentiated cells to retrodifferentiate to an undifferentiated cell(s).
70. A method or use according to any one of paragraphs 66-67 or 69 wherein said retrodifferentiating means is any means which causes negative selection within the cell

71. A method or use according to any one of paragraphs 66-67 or 69-70 wherein said retrodifferentiating means is any one or more of the following: an antibody; a density gradient medium used to separate cells according to the density of the cells; or a substance which causes sedimentation of red blood cells.

73. A method according to any one of paragraphs 68-69 or 72 wherein said relative free ion concentration of second environment is increased compared with the first environment.

75. A method according to any one of paragraphs 68-69 or 72-74 wherein said free ion is a group I or group II metal.

77. A method according to any one of paragraphs 68-69 or 72-76 wherein said free ion concentration of the environment is modified by treating said first environment with an agent capable of relatively changing the free ion concentration of the environment to effect said second environment.

79. A method according to any one of paragraphs 68-69 or 72-78 wherein said first environment is treated with one or more free ion sequestering agents and the cell population is subsequently transferred to a second environment, which second environment has an increased free ion concentration as compared with the first environment, thus effecting retrodifferentiation of the one or more differentiated cells in the cell population.

80. A method according to any one of paragraphs 68-69 or 72-78 wherein said cell population may be cultured in a first environment comprising a low or zero concentration of free ions followed by transferring the cell population to or adjusting the first environment so that it becomes a second environment comprising free ions or comprising free ions at a higher concentration than the first environment, thus effecting retrodifferentiation of one or more differentiated cells in the cell population.
81. A method according to paragraph 78-79 wherein said sequestering agent is a free ion chelating agent.
82. A method according to paragraphs 78-79 or 81 wherein said sequestering agent comprises both an amine and a carboxylic group.
83. A method according to any one of paragraphs 78-79 or 81-82 wherein said sequestering agent comprises a plurality of  $-N(CH_2CO_2H)_n$  groups, wherein  $n=1$  or  $n=2$ .
84. A method according to any one of paragraphs 78-79 or 81-83 wherein said sequestering agent may be selected from any one or more of the following: EDTA, heparin, EGTA, DTPA, trisodium citrate and other similar chelating agents and/or anticoagulants.
85. A method according to any one of paragraphs 78-79 or 81-84 wherein said sequestering agent is added in a sufficiently high concentration such that removal of the presence of said sequestering agent causes retrodifferentiation.
86. A method according to paragraph 85 wherein said concentration of the sequestering agent sufficient to cause retrodifferentiation when the presence of thereof is removed is more than or equal to about 2mM.
87. A method or use according to any one of paragraphs 66-86 wherein the more differentiated cells are non-cancer cells.
88. A method or use according to any one of paragraphs 66-87 wherein the more differentiated cells are committed haematopoietic cells.
89. A method or use according to any one of paragraphs 66-88 wherein the more differentiated cells are selected from CFC-T cells, CFC-B cells, CFC-Eosin cells, CFC-Bas cells, CFC-GM cells, CFC-MEG cells, CFC-E cells, T cells and B cells.
90. A method or use according to any one of paragraphs 66-89 wherein the undifferentiated cells are pluripotent stem cells.

91. A method or use according to any one of paragraphs 66-90 wherein the undifferentiated cells are stem cells from the group consisting of haematopoietic stem cells, neuronal stem cells, epithelial stem cells, mesenchymal stem cells, endodermal stem cells and embryonic stem cells.

92. A method or use according to any one of paragraphs 66-91 wherein the undifferentiated cells are characterised by one or more of the following cell surface marker designations: CD34<sup>+</sup>, HLA-DR<sup>+</sup>, CD38<sup>+</sup>, CD117, AC133, CD90 and/or CD45<sup>low</sup>.

93. A method or use according to any one of paragraphs 66-92 wherein the undifferentiated cells are MHC class I<sup>+</sup> and/or MHC class II<sup>+</sup> cells.

94. A method or use according to any one of paragraphs 66-93 wherein the cell population including committed cells is a buffy coat blood sample or is from a buffy coat blood sample.

95. A method of preparing an undifferentiated cell, the method comprising retrodifferentiating a differentiated cell in a cell population to an undifferentiated cell, wherein the environment comprising said cell population comprising one or more differentiated cells is changed from a first environment having a low or zero concentration of free calcium or magnesium ions to a second environment comprising free calcium or magnesium ions or comprising free calcium or magnesium ions at a higher concentration than the first environment, thus effecting retrodifferentiation of one or more differentiated cells in the cell population.

96. A method of preparing an undifferentiated cell, the method comprising retrodifferentiating a differentiated haematopoietic cell in a cell population to an undifferentiated cell, wherein the environment comprising said cell population comprising one or more differentiated haematopoietic cells is changed from a first environment to a second environment wherein the free ion concentration of said second environment is effectively modified as compared with the first environment so as to cause one or more of said differentiated haematopoietic cells to retrodifferentiate to an undifferentiated cell(s).

97. A method of preparing an undifferentiated cell, the method comprising retrodifferentiating a differentiated cell in a cell population to an undifferentiated cell, wherein the environment comprising said cell population is modified by treating a first

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